



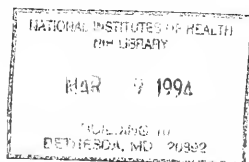


NATIONAL INSTITUTE OF  
DIABETES AND DIGESTIVE  
AND KIDNEY DISEASES

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DIVISION OF INTRAMURAL  
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INACTIVE PROJECTS

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Z01 DK 47007-17 DB

Z01 DK 48001-01 DB

Z01 DK 48003-01 DB

TERMINATED PROJECTS

Z01 DK 25073-04 LCB

Z01 DK 25081-02 LCB

Z01 DK 31107-05 LBC

Z01 DK 52018-01 GBB



## Annual Report of the

### Mathematical Research Branch

#### National Institute of Diabetes and Digestive and Kidney Diseases

Current research projects of the Mathematical Research Branch reflect a broad range of interests in the development and application of theoretical models and of quantitative methodologies for understanding biological systems.

This research involves several different collaborations within the Branch and with other research groups, both at the NIH and elsewhere. This report describes recent work in the areas of oscillatory activity of secretory cells, microcirculation, network and cellular neurobiology, renal physiology, and cell receptor aggregation. During the past year, international collaborative projects have involved foreign investigators at Hebrew University, Jerusalem (Department of Neurobiology), and at the University of Buenos Aires.

Invited presentations were given at distinguished symposia by J Rinzel (Annual Meeting, Neural Information Processing Society, Denver), by A Sherman (Annual Meeting, Society for Mathematical Biology, Ithaca; SIAM Conference on Applications of Nonlinear Dynamics, Snowbird) and by W Rall, J Rinzel, and I Segev (Bat Sheva Seminar on Neurobiology of the Motor System, Jerusalem). I Segev and J Rinzel initiated an interdisciplinary Computational Neuroscience Seminar series at the NIH. Several top scientists, each visiting for a few days, gave a talk (drawing wide attendance) and interacted extensively with interested groups. MRB staff were involved with various teaching activities. W Rall, as Honorary Lecturer, gave six lectures in the new course on Computational Neuroscience at Hebrew University. J Rinzel lectured and directed students at International Schools in Mathematical Biology in France and in Vancouver. I Segev, J Rinzel, A Sherman, and D Golomb were invited to teach in the course, Methods in Computational Neuroscience, Woods Hole, MA. P Frankel received his Ph D (Brown Univ, Applied Mathematics) for thesis research carried out at the NIH and directed by J Rinzel.

#### Oscillatory Activity of Secretory Cells.

Effects of electrical coupling among pancreatic beta-cells. We continue to explore the effects of cell heterogeneity on the electrical bursting and secretory activity of beta-cell populations by simulating clusters of 100 - 1000 cells with size, channel densities, and gap junctional conductances randomly distributed. Isolated model cells rarely burst because the parameter range for bursting is narrow, and most cells fall outside it. Coupled clusters burst, roughly, if the population average parameters are in the burst regime. This may explain why bursting oscillations are commonly seen in pancreatic islets, but rarely in isolated cells. Synchronized activity is obtained even if 1/3 of neighboring cell contacts are not electrical connections, as suggested by experiments. The model also argues against the hypothesis that recruitment of cells with heterogeneous glucose thresholds underlies the glucose dose response curve. In decoupled populations, recruitment produces only a shallow response. A sharp response requires synchronous activity mediated by non-linear coupling interactions. This agrees with some experimental comparisons of cell suspensions and islets. Further mathematical analysis of these phenomena is in progress using pairs of non-identical cells. (P Smolen, A Sherman, and J Rinzel)



We are complementing biophysical modeling studies of coupled beta cells with mathematical analysis of the dynamics of cell pairs. We decompose the system into fast, spike-generating, and slow subsystems and show how bifurcations of the fast subsystem affect bursting. When coupling is weak the spikes are out-of-phase and may even be aperiodic. We define the geometrical conditions under which this leads to substantially increased burst period, as reported in our large cluster simulations. These studies also suggest alternative ways that coupling can lead to bursting in cells that are not intrinsic bursters when uncoupled. In addition, we treat the fast subsystem by perturbation methods to derive a simplified, more easily analyzed set of equations. The method yields a complete mathematical characterization of the conditions for out-of-phase spikes and allows consideration of the effects of cell heterogeneity on the spike mechanism. (H-R Zhu and A Sherman)

Novel resetting of beta-cell burst rhythm. A longstanding, puzzling observation about bursting in pancreatic islets is that when the burst rhythm is reset from the silent phase to the active phase, or vice versa, by extracellular current pulses, the premature induced phase is of normal, rather than reduced, length. This is in contradiction to all models proposed hitherto. We have shown that this could be accounted for by a model with two slow variables, one to control the length of the active phase and one to control the length of the silent phase. If these variables recover rapidly in their off phase, the desired resetting behavior would obtain. A candidate channel exists for the plateau, but not the silent phase. The model suggests what properties the "missing channel" would require. (P Smolen and A Sherman)

Electrophysiological patch-clamp protocol. We have developed a new protocol in which patch-clamped HIT or beta cells are subjected to a bursting voltage waveform. This waveform is generated by a mathematical model (Sherman et. al., 1988) for beta-cell bursting and closely resembles bursts recorded from beta cells in intact islets. Pharmacological blockage was used to eliminate potassium and sodium currents in the clamped cells, and the calcium current during the waveform was monitored. In addition, brief pulses to a fixed depolarized voltage were superimposed upon the burst to enable the effect of calcium current inactivation to be separated from loss of activation. Both fast (hundreds of ms.) and slow (about 10 sec.) inactivation of calcium current was observed during the burst. The magnitude and timecourse of the slow inactivation is consistent with models of beta-cell electrical activity that assume the slow inactivation and recovery drives bursting. (P Smolen and L Satin: Medical College of Virginia)

Slow  $Ca^{++}$  oscillations in pancreatic beta-cells. Oscillations having periods about 5 minutes have been reported. Bursts on top of slow  $Ca^{++}$  waves and pure slow waves have both been seen, as well as oscillations with a similar period in electrical activity and metabolism. We test the hypothesis that these phenomena are related with a comprehensive model in which slow variations in ATP modulate the K-ATP conductance and hence  $Ca^{++}$  entry. We postulate an autonomous glycolytic oscillator which affects the rate of ATP synthesis, both later in glycolysis and in the mitochondria, assumed to oscillate in phase with the level of fructose biphosphate. Allosteric of phosphofructokinase and cycling of glucose-6-phosphate back to glucose are central features, leading to slow oscillations in intracellular glucose. Bursting and associated faster  $Ca^{++}$  oscillations are generated by faster modulation of the ATP synthesis rate in the mitochondria by  $Ca^{++}$ . Unlike other models for (fast)  $Ca^{++}$  oscillations, the ER plays a passive role here, but its uptake and release of  $Ca^{++}$  is essential to reproduce experimental timecourses. The model predicts that ATP and  $Ca^{++}$  oscillate in-phase, whereas antiphase oscillations would be expected if the main effect of ATP were activation of  $Ca^{++}$  removal. (P Smolen, A Sherman, and J Rinzel)



Calcium dynamics in pituitary gonadotrophs. A Hodgkin-Huxley (HH) like formalism is derived from a detailed kinetic model for oscillations of intracellular free calcium concentration, Ca. Our analysis reveals that the fast activation and slow inactivation of the InsP3-sensitive Ca-release channel InsP3-R by Ca are crucial for the occurrence of oscillations. We show that the experimentally observed "bell-shaped" Ca dependence of channel opening arises naturally as the "window" between the sigmoid Ca activation and inactivation curves. A simple two-variable model is obtained which retains the most important dynamic behaviors of the detailed kinetic scheme of Keizer, et al. (Y-X Li and J Rinzel) The analogy of our equations for Ca dynamics to the HH formalism were exploited in formulating a new model for Ca handling in pituitary gonadotrophs (experiments performed in lab of KJ Catt, ERRL/NICHD). By using generalized Boltzman expressions for the sigmoid Ca-dependence of channel activation and inactivation and by introducing appropriate regulatory effects InsP3 on these sigmoid curves, our model can reproduce a wide range of agonist-induced Ca responses and dose-dependent dynamic transitions observed in experiments. It shows that the transition from high frequency oscillations to the biphasic response is associated with the appearance of two stable steady states. Moreover, the persistence of our simulated oscillations until the pool is nearly depleted can be attributed to an additional mechanism, enhanced channel activation with decreasing ER luminal Ca (Ca-ER). Ca-ER regulation of activation sensitivity enables the late, second phase oscillations to occur. The ability of two chemical agents, thapsigargin and ionomycin, to generate oscillations is reproduced in a consistent way. The model also correctly predicts the response to combined actions of the 3 oscillation-inducing agents. (Y-X Li, J Rinzel, J Keizer:UC Davis, S S Stojilkovic:ERRB/NICHD)

#### Microcirculation.

Formulation and analysis of a model for vasomotion and vessel myogenic response has been completed (with B Ermentrout). It incorporates smooth muscle ionic transport of calcium and potassium, and the associated electrical activity and contraction of a thick walled cylinder of small artery dimensions. The model reproduces experimental responses to changes in intraluminal pressure (myogenic property). The formulation is being extended by incorporating several aspects of microcirculatory control: mechanical, neurohumoral and metabolic. The rheological effects of vasomotion on a microcirculatory network has been modelled. For some sets of rheological parameters, synchronization of the different arterioles is obtained; this suggests a mechanism for the similar phenomenon observed in sickle cell disease (with B Ermentrout) To study the behaviors associated with neuro-humoral controls (posture, hypertension), we have started to model agonist modulation of vasomotion and tone mediated by G-protein associated second messenger (with D Goldstein) Regarding metabolic control, we have developed a model that associates changes in tissue ADP/ATP with L-type Ca-channel and K ATP-sensitive channel activities. Different metabolic demands are shown to be associated with different vasomotion patterns. Interestingly, we find that the classical "capillary recruitment" is replaced by an "ensemble phase coincidence". (J Gonzalez-Fernandez, B Ermentrout:U Pittsburgh, and D Goldstein: U Buenos Aires)

#### Network Neurobiology

The lamprey central pattern generator for swimming. The isolated spinal cord of the lamprey can produce a travelling wave of neural activity as evidenced by repetitive bursts of action potentials recorded at the motor roots of each segment. The burst periods and the time delays between the bursts of different segments typically fluctuate about some mean. Correlations between the periods of different segments are positive and autocorrelations of delays one cycle apart are low. Using a stochastic model, we interpret these correlations as





suggesting that intersegmental coordination in the lamprey is strong and involves long distance connections. (A Cohen and N Mellon: Univ MD, and T Kiemel: Univ MD and MRB)

**Rhythmogenesis in thalamic networks.** Previously, we developed a model of globally coupled inhibitory neurons to explore Steriade's hypothesis that the reticular thalamic nucleus (RTN) is a pacemaker for sleep spindle oscillations. Our network of 10 excitable cells could oscillate in synchrony if the post-synaptic conductance decayed slowly enough, implicating the possible significance of GABA-B receptor-mediated inhibition (Wang and Rinzel, 1993). Now, considering a large network, we have described systematically several dynamical behaviors and the effects of various physiological parameters. In addition to a homogeneous fixed point and a homogeneous limit cycle, the system may exhibit cluster states, in which it breaks spontaneously into a few macroscopically large subgroups, each of which is fully synchronized (D Golomb and J Rinzel). A method for calculating the cluster states stability was developed and used to determine the regimes of existence of periodic attractors; the two-cluster states are described in detail. The effects of stochastic noise and heterogeneity amongst cells on network dynamics are also being studied (D Golomb and J Rinzel). When neuronal intrinsic excitability parameters are randomly distributed, the system exhibits four regimes of behavior. In addition to synchronized periodic and asynchronous regimes, we obtain two novel aperiodic regimes, with bursting rate a staircase-like function of neuron excitability. In one regime, the system is partially synchronized and in the second, partially antisynchronized. The transition between these two regimes is discontinuous as heterogeneity increases. Concurrent to these studies, we are refining our biophysical description of cells' intrinsic and synaptic ionic currents. Recent data on the T-type calcium current in RTN cells severely limits the maximal frequency of our model cells. However, now having incorporated distinct GABA-A and GABA-B components of synaptic inhibition, we are exploring their separate effects on the network's frequency. (D Golomb, X-J Wang: U Chicago, and J Rinzel)

**Visual information processing in the LGN.** We relate studies of the temporal coding of visual stimuli to measurements of the spatio-temporal receptive fields of neurons in the primate lateral geniculate nucleus (LGN). Our analysis includes 31 parvocellular and 10 magnocellular units. We use the framework of neural response, with the inclusion of output rectification, to predict the average temporal response of units to arbitrary stimuli. In general, we argue that there is no temporal code that relates the temporal behavior of neuronal output to the spatial pattern of the stimulus when the stimuli can vary in time. Measures of neuron reliability, and their decrease when the spike count is considered rather than the full temporal response, are estimated with the assumption of inhomogeneous Poisson statistics. Our estimates of mutual information agree with experimental results. (D Golomb, D Kleinfeld and BI Shraiman: Bell Labs, RC Reid: Rockefeller U)

**Epileptiform activity in hippocampal networks.** We have developed a "reduced" version of a detailed 19 compartment, 100+ variable model of a hippocampal neuron (Traub, et al, 1991). We have shown that our 8 variable, 2 compartment model replicates the salient features of the detailed model including a transition from repetitive bursting to repetitive spiking with increasing stimulation. Using a phase plane approach we are able to understand the mechanism of the repetitive bursting and spiking behaviors and to predict the effect of changes in parameters on the existence (and frequency) of bursting or spiking solutions. Traub et al showed using the detailed model of single neurons combined with NMDA and AMPA synapses that an excitatory network can generate multiple synchronized bursts. We have replicated this finding with an analogous network model utilizing our 2-compartment model neurons. Using qualitative insights gained from our model, we have developed an abstract model of an excitatory network. We have proven some results on



synchronization for this model and are currently doing simulation studies on biophysical and abstract excitatory networks to examine the robustness of synchronization in response to increasing cell variability. (P Pinsky and J Rinzel)

## Cellular Neurobiology

**Low-frequency firing and the potassium A-current.** We study the stimulus-frequency properties of the standard Hodgkin-Huxley (HH) model in the presence of a transient, potassium A-current, I-A. We challenge the folklore that low firing rates necessitate participation of an A-current. We gathered data (quite variable) on A-currents and determined that in most cases there was negligible steady state current. This suggested, and we showed, that I-A does not generally reduce to zero the firing frequency in the standard HH equations. Through transient effects, I-A does shift the onset of repetitive firing in HH and can promote period doubling oscillations. Also, I-A can shift the threshold for anodal break excitation and increase the latency to firing. Depending on the holding hyperpolarization level, break excitation may or may not occur. We found a "window" of excitation defined by the voltage range in which the two different potassium currents (the HH delayed rectifier and I-A) are negligible. The consequences of this are now being explored. (M Rush and J Rinzel)

**Bursting in a thalamic relay neuron.** We extended a quantitative model for low voltage, calcium-current, excitability (Wang, Rinzel, and Rogawski, 1991) by juxtaposing an HH-like model for sodium spiking in the high voltage regime to account for bursting. In the full model, we consider two types of burst responses: postinhibitory rebound burst and periodic bursting. In both cases, we found that an A-current shifts the threshold for sodium spikes (see above), and thereby reduces or eliminates spikes from the burst. It can also annihilate periodic bursting in some parameter ranges. Bursting modulation may be linked to the hyperpolarizing/depolarizing effects reported in thalamocortical neurons and reticular thalamic neurons after application of certain neurotransmitters. We explore the ionic mechanisms for this and confirm some experimental findings. (M Rush and J Rinzel)

**Dendritic signal processing.** Input location, input timing and chain reactions of spike firing in excitable dendritic trees were explored by means of computer simulations. These results demonstrate (for dendrites that receive different distributions of synaptic excitatory and inhibitory inputs) that the presence of excitable ion channels in the dendritic membrane can cause the dendritic tree to fractionate into many subregions; each of these subregions can function as a coincidence detector in the sub-millisecond range. (W Rall and I Segev: Hebrew Univ and MRB)

**A novel analytical method to analyze signal delay in dendrites has been created and tested.** It was demonstrated that dendritic trees provide for a communication line with multiple time scales. For computations that take place locally in distal dendrites, (e.g. dendro-dendritic synaptic interactions, or the triggering of plastic change), the critical time-window is on the order of 0.1 in units of the passive membrane time constant,  $\tau$ . For communications over longer distances, (e.g. to the axon from synaptic input locations), the meaningful time scale is larger, on the order of 1.0 in units of  $\tau$ . (H Agmon-Snir: Hebrew Univ and I Segev: Hebrew Univ and MRB)

## Renal Physiology.

**Role of the kidney in acid/base balance.** We have continued development of a multinephron model for acid/base balance in the whole kidney as experiments to measure the necessary parameters have continued. Permeabilities for ammonium and bicarbonate of chinchilla and rat thin ascending limbs of the loop of Henle have now been measured and



used in the model. Results obtained with the model using measured permeabilities and interstitial profiles are consistent with countercurrent trapping of ammonium in the renal medulla that involves mainly  $\text{NH}_4^+$  transport. With detailed modeling we have described the contributions made by different nephron segments and medullary regions. Concurrently, a database (set in MEDLINE format and to be widely available) of renal parameters is being developed as we extend the model to include distal segments of the nephrons. (R Mejia and M Knepper:NHLBI)

**Concentrating mechanism.** In vitro measurements in rodents have shown osmotic water permeability to decrease from the upper long descending limb of Henle in the outer medulla to the thin ascending limb, and both  $\text{NaCl}$  and urea permeability to increase. While measurements of tissue osmolality have shown approximately equal concentration profiles in inner and outer medulla, present models of urinary concentration concentrate minimally in the inner medulla with these permeabilities. Numerical experiments, conducted to study possible mechanisms in the inner medulla, have shown that single effects in the descending limbs, ascending limbs and collecting duct that could be available for countercurrent multiplication are not compatible with experimental measurements. This suggests consideration of models that incorporate more detail of kidney function (e.g. dynamics), although to date no prime candidate has arisen. (R Mejia, C-L Chou and M Knepper:NHLBI)

**Cellular responses to receptor aggregation.**

Early events in allergic reactions. In immediate hypersensitive allergic reactions, the aggregation of Fc-epsilon receptors on basophils and mast cells leads to the release of histamine from intracellular granules. Previous theoretical and experimental collaborative work has clarified the relation between ligand binding, receptor aggregation and both the activation and desensitization of basophils and mast cells. This work has also revealed differential effects of aggregates of distinct sizes and structures. Recent experimental advances permit one to observe a response that occurs much earlier than histamine release in the signaling cascade, i.e., the phosphorylation of tyrosine residues on receptor subunits and other cellular proteins. In the current collaborative project, the ligand used to crosslink receptors was chosen because it induces the formation of well defined receptor aggregates that do not break up over the course of the experiment. The system is particularly suitable for modeling (and extracting quantitative information from the model-based data analysis). An early and surprising finding is that receptors and other cellular proteins remain phosphorylated for a long time (on the order of hours). Based on earlier experiments where crosslinking is reversed and cellular responses stopped, it had been argued that cellular responses are short-lived and depend on continued formation of new crosslinks. (B Goldstein: Los Alamos Natl Lab, MRB and NIAMS, Carla Wofsy: U New Mexico, MRB and NIAMS and H. Metzger: NIAMS)

**Cytokine receptor aggregation.** Many cytokines initiate signaling cascades by inducing receptor aggregation. Some, such as granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 3 (IL-3) and interleukin 5 (IL-5), share a common nonbinding signaling subunit, Beta. To dissect this system and begin to understand the nature of the competition and synergy among subunits, binding studies were performed on cell lines transfected with one or more of the proteins of interest (receptors and signaling subunit). Initial analysis by the experimentalists, using a standard model that did not include receptor aggregation, masked information that we were able to extract using a model that includes aggregation. An analysis of earlier data and new experiments, comparing two distinct aggregation models is in progress. In one model, aggregation occurs by the ligand binding simultaneously to the receptor and the signaling unit, crosslinking them. In the alternative model, subunits pre-aggregate to form high affinity



receptor. (B Goldstein: Los Alamos Natl Lab, MRB and NIAMS, Carla Wofsy: U New Mexico, MRB and NIAMS and T Kitamura: DNAX Res Inst).





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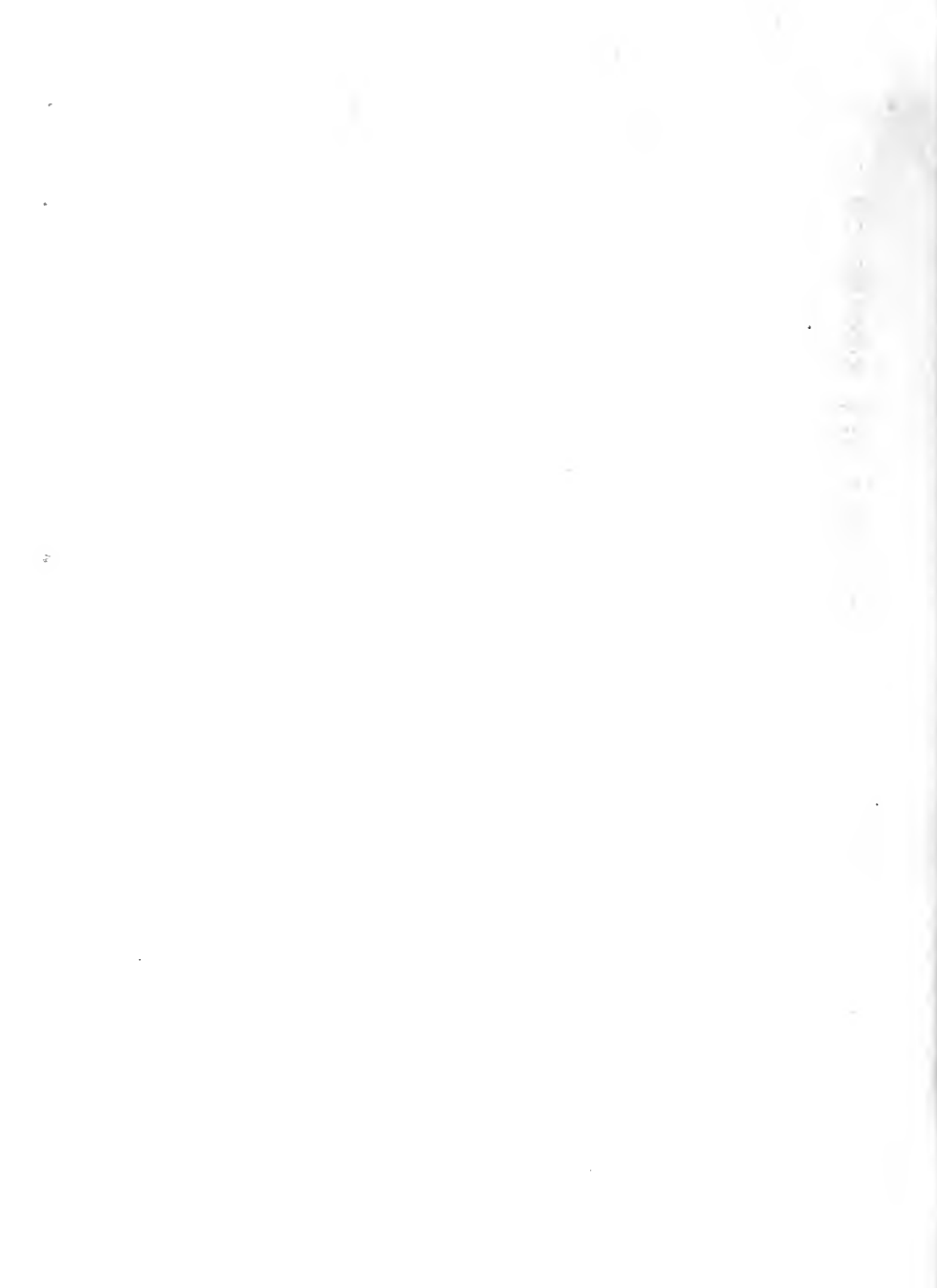
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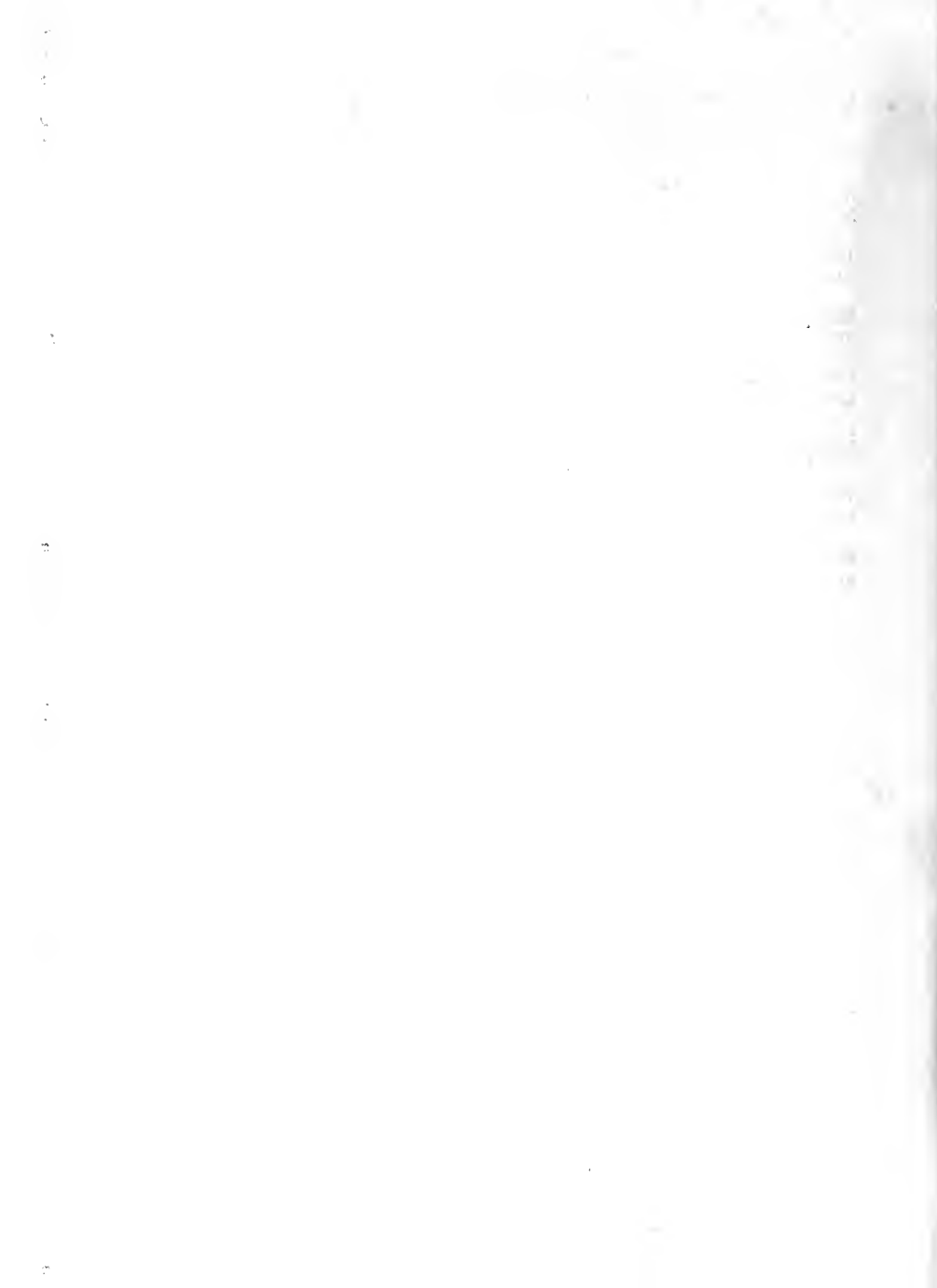
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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13001-20 MRB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical formulations and analysis relevant to experimental neurophysiology.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W. Rall

Senior Research Physicist

MRB, NIDDK

Others: I. Segev

Fogarty Visiting Scientist

MRB, NIDDK

## COOPERATING UNITS (if any)

Dept. of Neuroscience, Hebrew Univ. of Jerusalem

## LAB/BRANCH

Mathematical Research Branch

## SECTION

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.3

PROFESSIONAL: 2.0

OTHER: .3

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**RESEARCH AREA.** Basic neuroscience involving structure/function relations for neuronal dendritic branching, dendritic spines, and synapses (also neuron populations, with cortical symmetry), and for such functions as synaptic transmission, amplification and dendro-dendritic interactions in the context of spatio-temporal input patterns, logical processing of input, and neural plasticity, as in conditioning and learning.

**RATIONALE.** Combine experimental data from neuroanatomy and from electrophysiology with biophysical models of nerve membrane (passive, synaptic and excitable) into a comprehensive theory which can lead to new insights and to testable theoretical predictions (leading to the design of better experiments). To do this, we must create, explore and test mathematical and computational models with different degrees of complexity.

**METHODOLOGY.** Our methods include both analytical solutions and computational solutions of boundary value problems (for partial differential equations) in the traditions of classical physics. They include also the formulation and solutions of problems in terms of systems of ordinary differential equations; when this is done explicitly for a compartmental model of a neuron, it is possible to accommodate a remarkable variety of dendritic branching patterns and non-uniform distributions of membrane properties and of synaptic inputs.

**RESULTS.** Perspective, results and references can be found in a recent review and in chapters in several recent books: see the Oct 1992 special issue of Physiological Reviews, 72: S159-S186; see also "Single Neuron Computation: (T. McKenna, J. Davis and S.F. Zornetzer, eds) Academic Press, 1992, and "Computational Neuroscience" (E.L. Schwartz, ed.) MIT Press, 1990.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13002-21 MRB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical Description of Substrate Transport to Capillary-Tissue Structures

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Jose Gonzalez-Fernandez

Research Mathematician

MRB, NIDDK

Others:

## COOPERATING UNITS (if any)

Bard Ermentrout (University of Pittsburgh), Daniel Goldstein (University of Buenos Aires School of Sciences)

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 1.33

PROFESSIONAL: 1.3

OTHER: .03

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A formulation for vasomotion and vessel myogenic response written in collaboration with Bard Ermentrout, University of Pittsburgh, has been completed. It incorporates smooth muscle ionic transports-calcium and potassium-and the associated electrical and contraction of a thick walled cylinder of small artery dimensions. The model reproduces the experimental responses to changes in intraluminal pressure (myogenic property). The analysis is contained in the paper "On the Origin and Dynamics of the Vasomotion of Small Arteries," Mathematical Biosciences, in press.

The formulation is being extended with the purpose of incorporating several aspects of the microcirculatory control: mechanical, neuro-humoral and metabolic.

The rheological effects of the vasomotion on a microcirculatory network has been modeled. For some sets of rheological parameters, synchronization of the different arterioles is obtained; this suggests a mechanism for the similar phenomenon observed in sickle cell disease (with Bard Ermentrout, University of Pittsburgh).

To study the behaviors associated with neuro-humoral controls (posture, hypertension), we have started to model agonist modulation of vasomotion and tone mediated by G-protein associated second messenger (with Daniel Goldstein, University of Buenos Aires, School of Sciences).

We have developed a model that associates changes in tissue ADP/ATP with L-type Ca-channels and K ATP-sensitive channels activities. Different metabolic demands are shown to be associated with different vasomotion patterns. A feature of interest is that the classical "capillary recruitment" is replaced by an "ensemble phase coincidence".





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13004-20 MRB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical Description of Cellular Neuroelectric Signal Transmission

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

<b>PI:</b>	John Rinzel	Chief	MRB, NIDDK
<b>Others:</b>	Arthur Sherman	Senior Staff Fellow	MRB, NIDDK
	Paul D. Smolen	NRC Fellow	MRB, NIDDK
	Yue-Xian Li	Visiting Fellow	MRB, NIDDK
	David Golomb	Visiting Fellow	MRB, NIDDK
	Maureen Rush	Pre-doc IRTA	MRB, NIDDK
	Paul Pinsky	Pre-doc IRTA	MRB, NIDDK

## COOPERATING UNITS (if any)

I. Atwater, E. Rojas (LCBG, NIDDK), S. Stojilkovic (ERRB, NICHD), L. Satin (Virginia Comm. Med. School), X.-J. Wang (Univ. Chicago), J. E. Keizer (Univ. California, Davis)

## LAB/BRANCH

Mathematical Research Branch

## SECTION

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 4.5

PROFESSIONAL: 4.0

OTHER: 0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project continues to focus on the formulation, analysis, and biophysical interpretation of mathematical models which describe various aspects of electrical activity of neurons and other cell types. Among the topics of current interest are: (i) integration of synaptic input delivered to the soma and dendritic branches of a neuron; (ii) propagation of action potentials along axons; (iii) stimulus-response and threshold properties for repetitive-firing of action potentials; (iv) complex bursting patterns of membrane potential oscillations which arise through endogeneous membrane properties and/or intercellular coupling.

Because qualitatively related mathematical or biophysical problems may arise in other context, e.g., chemical and biochemical oscillations, or e.g. excitation-secretion coupling, this project may consider models from such applications.

Mathematical models of these phenomena involve systems of linear and nonlinear ordinary differential equations and parabolic partial differential equations. Solutions and their mathematical stability are determined by analytical and numerical methods drawn from both classical and modern applied mathematics. These methods may include finite difference or finite element numerical integration, bifurcation theory, perturbation methods, and nonlinear dynamical systems theory. One goal of this project is to expose the qualitative mathematical structure for classes of models by exploiting simple, yet physiologically reasonable, equations.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13020-04 MRB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Electrical and Chemical Oscillations in Coupled Cell Systems

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

<b>PI:</b>	Arthur Sherman	Senior Staff Fellow	MRB, NIDDK
<b>Others:</b>	J. Rinzel	Chief	MRB, NIDDK
	M. Mascagni	Guest Worker	MRB, NIDDK
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## LAB/BRANCH

Mathematical Research Branch

## SECTION

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.0

PROFESSIONAL: .1

OTHER: 2.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We use mathematical models to study the mechanisms of oscillatory electrical activity arising from ion channels in cell membranes and modulated by intracellular chemical processes. We are interested in both the behavior of single cells and the ways in which cells communicate and modify each other's behavior.

Our main application has been to the biophysical basis of insulin secretion in pancreatic beta-cells. We have examined bursting oscillations in membrane potential and the role of electrical coupling between cells in the islet of Langerhans. Long term goals are to understand how the membrane dynamics interact with intracellular events to regulate secretion and to generalize to other secretory cells and neurons.

Our primary tool is the numerical solution of ordinary and partial differential equations. We use analytical, geometrical, graphical, and numerical techniques from the mathematical theory of dynamical systems to help construct and interpret the models. Perturbation techniques are used to get analytical results in special cases.

We study both detailed biophysical models and simplified models which are more amenable to analysis. Such an approach aids the isolation of the essential or minimal mechanisms underlying phenomena, the search for general principles, and the application of concepts and analogies from other fields.

We see a role for our group as intermediaries between the mathematical and biological disciplines. This includes disseminating the insights of mathematical work to biologists in accessible language and alerting mathematicians and other theoreticians to new and challenging problems arising from biological issues.

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## REPORT OF THE LABORATORY OF CELLULAR AND DEVELOPMENTAL BIOLOGY

The forty scientists of LCDB focus on study of the critical elements of development and differentiation: how extracellular signals are translated, through transducers and second messengers, into differential gene expression in the nucleus, and how the architecture of nucleic acids and proteins is involved in nuclear and cytoplasmic function. This is an exciting area of modern biology and one in which members of the laboratory are widely recognized as key players.

Several noteworthy recognitions have come to laboratory personnel this year. Dr. Jurrien Dean received the USPHS Outstanding Service Medal. Dr. Dean is a member of the NIH Biosafety Committee. Dr. Alan Kimmel serves as a member of the NIH Committee for Molecular Medicine. Dr. Charles Schultz received his Ph.D. from the University of Illinois and moved from Dr. Robert Scow's group to a post-doctoral fellowship with Dr. Dean Londos. Dr. Londos is lead cochair for a Keystone meeting next winter on the Adipocyte, an extension of the successful meeting he organized two years ago. Dr. Robert Simpson is a member of the Director's Reserve for Study Section members and presented LCDB studies at the Cold Spring Harbor Symposium on Quantitative Biology, entitled "DNA and the Chromosome," in June. Many members of the laboratory presented their work at other major meetings and lectured at NIH, universities, institutes and commercial firms.

Several organizational changes have taken place in LCDB in the past year. Dr. Bernard T. Kaufman retired from NIH in June, after more than 30 years of investigations of the metabolism of folates and dihydrofolate reductase. His presence will be missed by those of us who have known Toby for many years and we wish him well in his retirement. The Morphological Studies Unit in the Office of the Chief, LCDB, has been established with Dr. Joan Blanchette-Mackie as its Head. Dr. Blanchette-Mackie is a major collaborative resource for scientists within and out of LCDB, using her expertise both in electron microscopy and with a recently acquired confocal microscope. The Section on Molecular Mechanisms of Development was established with Dr. Alan Kimmel as its Chief. This Section studies the transduction of extracellular signals to differential gene expression in the simple eukaryote, *D. discoideum*. Its formation is a recognition of the major contributions made by the Kimmel group to understanding of principles governing developmental gene regulation.

The review of the laboratory's activities during the past year is not organized by Section or working group, but thematically. This approach provides the reader with an overview of the laboratory that emphasizes the continuity in research interests, a continuity which exists in spite of the broad span of areas of modern biology investigated in the Laboratory of Cellular and Developmental Biology.

### **Molecular mechanisms of gene regulation**

Clearly, gene regulation is of critical importance in both normal development and disease. Increasing evidence suggests that in some cases the same DNA sequences are differentially expressed depending on their chromosomal localization and/or chromatin structure. Genetic



imprinting, position effect variegation, epigenetic inheritance and silencing of X chromosomes, telomeric and centromeric regions are all likely candidates for effects of chromatin structure on transcriptional regulation. Other work indicates that similar chromatin effects on other functions of DNA, replication, recombination and repair, are likely.

Nucleosome positioning has been suggested to be a possible mechanism in determining the function of *cis*-acting elements in eukaryotic cells. Previously, we showed that movement of a *cis*-acting element necessary for yeast replication origin function into the center of a nucleosome markedly diminished its accessibility to *trans*-acting factors. Similarly, we showed that a yeast repressor organized positioned nucleosomes abutting its operator, placing the TATA box in the center of a nucleosome, and suggested that this might be the mechanism of repression of the  $\alpha$ -cell specific genes which are repressed by the  $\alpha 2$  repressor protein. We have extended our investigations and now find that at least four precisely positioned nucleosomes are located adjacent to the *STE6*  $\alpha 2$  operator in  $\alpha$ -cells. They are organized as two pairs of close packed dimers separated by a 40-45 bp linker. This has interesting structural consequences in terms of potential interactions of the dimers with other proteins in creating a chromatin domain. The domain is organized in an active fashion by the  $\alpha 2$  repressor. Studies with histones and isolated DNA fragments *in vitro* failed to demonstrate any preferred location for histone-DNA interactions that reflected the *in vivo* situation. Further, insertion of 75 or 200 bp of different DNA adjacent to the  $\alpha 2$  operator led to maintenance of the location of the positioned nucleosome, even though its DNA context was completely different.

We postulated that the mechanism of repression of  $\alpha$ -cell specific genes by the  $\alpha 2$  repressor is establishment of a repressive chromatin structure wherein the TATA element is placed in an inaccessible location, in the center of a positioned nucleosome. Critical experiments to test this hypothesis have been performed. Others have recently shown that a heteromeric protein formed by the products of the *SSN6* and *TUP1* genes is necessary for repression by  $\alpha 2$ . We disrupted each of these genes and found that the positioned nucleosomes near the  $\alpha 2$  operator were not present in either the genome or nontranscribed minichromosomes in the null strains. Topoisomer distributions were also different; compared to wild type  $\alpha$ -cells, minichromosomes had about 1 or >2 fewer negative supercoils in a *ssn6*- or *tup1*-background, respectively. These observations make the Ssn6p/Tup1p heteromer a strong candidate for a bridging protein in the interactions between  $\alpha 2$  and histones which establish the organized chromatin structure. The product of the *SIN4* gene is also necessary for  $\alpha 2$  repression. In a *sin4*- background, positioned nucleosomes about the  $\alpha 2$  operator but are seen only at very low levels of nuclease digestion, suggesting that they are perturbed in a fashion that makes them highly unstable.

A definitive test of the location of the TATA box in the middle of a positioned nucleosome as a repressive mechanism was carried out by inserting increasing numbers of 25 bp lengths of DNA between the operator and TATA box of the *STE6* promoter controlling a reporter gene. In  $\alpha$ -cells, increasing the distance between the two elements lead to a monotonic decrease in transcription. No expression was observed in  $\alpha$ -cells, even in the 75 bp insertion which places the TATA box in a short linker next to the nucleosome positioned by the  $\alpha 2$





repressor. The *STE6* promoter also contains two Ste12p pheromone response elements which may facilitate transcription complex assembly. Their location is such that none of the insertion mutations would have allowed both the TATA box and the Ste12p binding sites to be exposed simultaneously. Mutation of the Ste12p sites led to only a 3-4 fold reduction in transcription, making it unlikely that their inaccessibility led to absence of transcription in  $\alpha$ -cells. It seems more likely that the repression derives from the organized domain of chromatin rather than a single nucleosome. Formation of the basal transcription complex leads to a 80 bp footprint on DNA; the short linker probably does not allow sufficient proteins to interact and disrupt the adjacent nucleosome.

In contrast, we previously reported that location of the intragenic control elements of a tRNA gene, transcribed by RNA polymerase III, in a predicted positioned nucleosome led to fully active transcription. The predicted nucleosome was not present; the Pol III transcription factors dominated. We now find a similar situation for a Pol II gene controlled by GAL4 protein. The protein can bind and activate transcription when its binding site is located in a predicted nucleosome. Chromatin mapping shows that the predicted nucleosome is perturbed when GAL4p is present, either in galactose or expressed from a constitutive promoter in glucose, but present in the anticipated position when GAL4p expression from its own promoter is repressed in glucose. Binding of GAL4p does not require the acidic activation domain; it is observed with a truncated protein containing only the DNA binding domain or when Gal80p masks the acidic domain of GAL4p constitutively expressed in glucose. However, perturbation of adjacent nucleosome structure, particularly one containing a TATA box, is dependent on both the TATA element and the Gal4p activation domain, suggesting that recruitment of the preinitiation complex is a role of Gal4p. The binding of GAL4p is dominant over a nucleosome positioned by a DNA sequence based mechanism or positioned by the  $\alpha 2$  repressor. Clearly hierarchies exist in the interactions of *trans*-acting factors with chromatin and we are only beginning to start to sort out the rules that govern the competition. Areas of interest for future studies include the timing of association of factors with DNA relative to replicative assembly of chromatin and the role of other gene products, such as the Swi proteins, which are necessary for certain *trans*-activating proteins to contend with chromatin.

In diploid cells, both  $\alpha$  and a mating type information are expressed and a heterodimer of two homeodomain containing proteins,  $\alpha 1$  and  $\alpha 2$ , represses expression of a series of haploid specific genes. One of these genes, *SST2*, is flanked by three  $\alpha 1/\alpha 2$  operator like sequences, 450 to 700 bp 5' of the structural gene. The central one of these appears, in preliminary studies, to be flanked by positioned nucleosomes extending towards the structural gene. This suggests that the heterodimer may in some cases use a similar strategy for repression as does the  $\alpha 2$  homodimer. We say in some cases since a first examination of the 5' flanking region of another haploid specific gene, *RME1*, did not show an organized chromatin structure. Clearly, more extensive investigations, parallel to those carried out for  $\alpha 2$  repression, will be necessary to establish mechanism and generality.

Protein-DNA interactions at the  $\alpha 2$  operator (and other specific loci) *in vivo* are of high interest in terms of understanding all the factors that interact with DNA and for comparison with structures deduced from *in vitro* reassembly using purified components. Others have



previously utilized prokaryotic *dam* methyl transferase to modify DNA in *S. cerevisiae*. GATC sites are methylated at N6 of adenine by this enzyme; wild type yeast lack 6-methyladenine. We have now carried out a systematic investigation of the accessibility of DNA in nucleosomes, linkers and nuclease hypersensitive regions to the methylase, an investigation which has not been done by others who have loosely interpreted accessibility in terms of "open" or "closed" chromatin structure. We constructed two derivatives of the well characterized TALS plasmid containing a total of twelve GATC sites - one in a hypersensitive region, one in a linker, and ten placed at various locations and in different rotational settings within a positioned nucleosome abutting the  $\alpha 2$  operator. A novel quantitative slot blot assay was developed to measure the extent of methylation at each site. The linker region is accessible in either  $\alpha$  or  $\alpha$  cells. The level of *dam* accessibility declines markedly in  $\alpha$  cells between 21 and 31 bp from the edge of the nucleosome; sites from 31 bp in to the pseudodyad of the core particle are completely refractory to methylation in  $\alpha$  cells. These same sites are frequently dimethylated in  $\alpha$  cells, where the  $\alpha 2$  repressor is absent and the positioned nucleosome is absent or randomized in its location. Rotational setting (major groove facing out to solution or in to octamer) did not affect either the peripheral accessibility of *dam* sites or the central inaccessibility of these sites. The GATC sequence in the ARS1 origin of replication is methylated in about half of the minichromosomes. Although this is a nuclease hypersensitive region, it is apparent that other proteins, besides histones, can prevent access of the methylase to DNA; presumably, in this case it is the recently identified origin recognition complex. These results are of importance in establishing a benchmark for use of this powerful approach to study of chromatin structure *in vivo*. Under consideration is extension of the methylation approach to chromatin structure in two areas; (i) controlled expression of the *dam* methylase to allow study of cell cycle related remodelling of chromatin and (ii) use of a more ubiquitous enzyme which methylates CpG sequences.

Other studies in the laboratory address directly the role of *cis*-acting DNA elements and *trans*-acting factors in regulation of gene expression. The most studied system in this context is the heat shock gene *HSP82*, a gene whose expression is also induced during meiosis. Experiments in the last year, using a single copy *HSP82-lacZ* fusion integrated into the genomic *HSP82* locus, have defined the DNA sequences required for meiotic induction of the gene. A 112 bp segment of the gene including the TATA box at -78 and the heat shock element (HSE) at -176 is necessary and sufficient for meiotic and mitotic expression. Substantial meiotic induction is obtained when the HSE is replaced with HSEs from genes which are not induced during sporulation; the HSE is required for induction but the specificity of meiotic induction resides elsewhere in the DNA fragment.

A second *cis*-acting element has been identified between the TATA box and the HSE, residing at -112 to -137. When this region is mutated, a 3-4 fold increase in basal expression of a reporter gene is observed. Within this region, there is a 10 bp motif that corresponds to a widely found repressor sequence, URS1. This motif, when mutated, leads to increases in basal expression. Similar to the situation for HSE activation, the flanking sequences about URS1 are important for its function in its native context; mutation of abutting sequences leads to the full extent of derepression seen in core element mutants. Dissection of the *cis*-



acting elements important for the sporulation expression of the *HSP82* gene will continue as a prelude to the search for the *trans*-acting factors that mediate this response.

A number of mammalian genes whose expression is developmentally regulated are under investigation in the laboratory. We are investigating the tissue specific and developmental stage specific expression of the human  $\epsilon$ -globin gene, the first of the  $\beta$ -like globin genes to be expressed during development. Recent studies in transgenic mice have shown that the gene promoter, under the control of the  $\beta$ -globin locus control region (LCR), contains all the information necessary for correct developmental expression of the human  $\epsilon$ -globin gene. The  $\beta$ -globin LCR exhibits two kinds of properties; it has long range effects on the chromatin structure of the  $\beta$ -globin locus and it acts as a powerful enhancer. Current data suggest that these properties result from complex interactions between transcription factors and the promoter and enhancer DNA sequences.

In previous studies, nuclear factor binding sites were mapped in the promoter of the  $\epsilon$ -globin gene and in the  $\beta$ -globin LCR HS II enhancer that increases activity of the promoter by 100-fold. Our studies also demonstrated that enhancer-promoter communication depended on interactions of two erythroid specific proteins with these regulatory elements, GATA-1 with the promoter, and NF-E2 with the enhancer. Mutations in these elements eliminated enhancement in an all or nothing fashion. We had also observed that the ubiquitous transcription factor Sp1 bound to the CACCC site (-110) of the promoter, and to two CACCC sites in the enhancer, flanking the tandem AP-1/NF-E2 binding sites. We individually mutated these binding sites and found that Sp1 also participates in enhancer mediated transcription from the  $\epsilon$ -globin promoter. Elimination of binding of Sp1 to the promoter CACCC site reduced activity to about 25% of the enhanced level, and mutation of the Sp1 site 5' to the enhancer NF-E2 motif (but not the site that is 3') reduced promoter activity to 70% of the enhanced level. These results suggest that action of the  $\beta$ -globin LCR on the  $\epsilon$ -globin promoter to increase transcription reflects the DNA binding activity of an apparently ubiquitous protein(s) that recognizes CACCC elements (Sp1), as well as erythroid restricted proteins GATA-1 and NF-E2 that bind GATA and AP-1 motifs in the promoter and enhancer, respectively. The role of Sp1 has been suggested to include looping of DNA to facilitate interactions between regulatory proteins bound at long distances on DNA; the results of this work are consistent with such a role.

We have begun an analysis of the role of the enhancer in transcriptional regulation both *in vitro* and *in vivo*. Abundant transcription was directed by naked DNA templates in erythroid as well as non-erythroid cell extracts. This presumably reflects the lack of tissue specific promoter repression of naked DNA templates. The presence of the  $\beta$ -globin LCR HS II enhancer did not further increase transcription. Histones from chicken red cells were deposited on the templates by salt dialysis at ratios of 0.8 and 1.2 histone protein to DNA. Topoisomer gels indicated the presence of up to 20 nucleosomes per plasmid. In the absence of the enhancer, assembly with histones repressed transcription to about 50% of the level observed for naked DNA templates. When the enhancer fragment was present in the template, transcription was only about 15% lower than for naked DNA. The enhancer acts as what others have called an antirepressor.



*In vivo*, we use an episome based on the EBV origin of replication and nuclear antigen to study the interaction of erythroid enhancers and promoters in chromatin. The episome is stably maintained at copy numbers between 2 and 200 in several human tissue culture cell lines and is assembled into chromatin. In earlier studies we inserted a marked  $\epsilon$ -globin gene into the episome. In both nonerythroid and erythroid cell lines, no transcription from the episomal  $\epsilon$ -globin gene was detected. We have now made a new series of episomes that contain the  $\epsilon$ -globin gene without or with the  $\beta$ -globin LCR HS II enhancer fragment, or the  $\mu$ LCR, a fusion of the four  $\beta$ -globin LCR HS sites. In the absence of any enhancer only the chromosomal  $\epsilon$ -globin gene is transcribed. However, in all clones of cells containing enhancer fragments, we observed substantial transcription from the episomal  $\epsilon$ -globin gene. Enhancer dependent transcription from the episome will permit studies of the relation between structure and function of this gene and has the potential of providing a means to assay the locus opening activity of the  $\beta$ -globin LCR.

Genes encoding the proteins which form the zona pellucida that surrounds growing oocytes, function as primary and secondary sperm receptors and protect the preimplantation embryo are tissue specific and developmentally regulated. Two of these genes, *ZP2* and *ZP3*, have been cloned and studied in this laboratory from both human and murine sources. Putative regulatory *cis*-acting sequences have been shown to be conserved for both genes in both species and necessary for the developmentally regulated expression of the zona genes.

We have identified a putative transcription factor (*ZAP-1*) that binds to this DNA motif. We presume that binding of *ZAP-1* plays a role in the coordinate and oocyte specific expression of the zona genes. The *ZAP-1* DNA binding activity is first detectable in oocytes from 19 day mouse embryos and reaches a maximal level at 10 days after birth. This developmental profile closely parallels that for mouse zona protein expression. The complex is detected in mouse, rat, opossum and human (but not *Xenopus*) ovarian extracts, suggesting conservation of the presumed regulatory protein among mammals. A concatenated oligonucleotide containing the binding site of *ZAP-1* has been used to screen a random primed cDNA library. A clone containing a sequence which is unknown to GenBank was isolated and its expression shown to be restricted to oocytes. There is a single open reading frame in the partial cDNA encoding an unusual 214 amino acid polypeptide that consists of a 40 amino acid motif repeated five times. Each repeat contains three acidic residues spaced eight amino acids apart followed by five basic residues spaced two to four amino acids apart. Within these charged residues, there is a high content of prolyl and glycylic residues, suggesting the absence of organized secondary structure for the protein.

### **Cell biology of developing systems**

While certain aspects of LCDB research also concern molecular levels of gene regulation, they are more focussed on the organismic aspects of development as a long term goal for understanding. In this area, studies of the development of *Dictyostelium* and studies of organ development, particularly gonadogenesis in the mouse, are examples of our research.

Mouse gestation is complete 20 days after fertilization. The primordial germ cells, first identified 7.5 days post-coitum (dpc), complete a migration from the allantois to the genital





ridges by 12.5 dpc. The expression of the *Sry* gene (testis determining factor on the Y chromosome) in support cells beginning at 10.5 dpc is associated with the differentiation of the gonad into the testis by 12.5 dpc. In female mice, ovaries can be detected morphologically by 13.5 dpc.

A mRNA differential display technique is being employed to identify genes that are either causal or a result of differentiation of the primitive gonad into the ovary. Genital ridges from 10.5 to 12.5 dpc fetuses were isolated by dissection. The ridges were separated into male and female pools after their sex was determined by PCR using oligonucleotides specific to the *Sry* gene. Single strand cDNA was made and amplified using PCR with an anchored and random oligonucleotide primers. Bands that were sex specific on a sequencing gel analysis were cut out and reamplified. Two previously unreported female specific mRNA sequences have been identified thus far; further characterization of these genes is proceeding. This strategy is a rational approach to study of genes important in organogenesis; study of the reproductive system has the virtue that mutations in genes necessary for organ formation will not be lethals, as they would for most other organ systems.

Assembly of the zona pellucida is an important feature of oogenesis and one which we wish to study both *in vivo* and *in vitro*. One persistent problem has been lack of the gene for ZP1. We have now obtained protein sequence information from HPLC separated tryptic peptides of gel purified ZP1. Three short peptides have a 50-75% match with sequence from rabbit rc55 and pig ZP3 $\alpha$ , two homologous zona proteins that are neither ZP2 nor ZP3. An oligonucleotide probe based on the rc55 sequence that encodes 16 amino acids that are 80% identical among the three proteins is being used to screen mouse ovarian cDNA libraries to isolate the ZP1 gene.

The three zona proteins are secreted and interact to surround growing oocytes with an extracellular coat. Microinjection of specific antisense oligonucleotides into oocytes causes degradation of either ZP2 or ZP3 transcripts. New zona protein synthesis is abolished for the targeted mRNA but the other zona protein continues to be made. Inhibition of synthesis of either ZP2 or ZP3 prevents incorporation of the other protein into the extracellular zona matrix; whether the second zona protein is secreted or degraded is not known. Microinjection of the cognate human protein is being used to try and restore zona assembly and glean information about the regions of the proteins which are important for their interactions. An important and ongoing extension of this work is study of the same processes *in vivo*. We have established conditions for propagation and mutation of embryonic stem cells. To disrupt the *Zp-2* and *Zp-3* genes, we have isolated each gene from an isogenic murine library. Once knock out cells are obtained, they will be used to construct mouse lines with null mutants for each gene for study of folliculogenesis and early development.

Cyclic AMP is an important, pleiotropic effector of development for *Dictyostelium*, serving as a paracrine hormone. Development is dependent upon signal transduction mediated by cell surface, cAMP receptor/G protein linkages. Secreted cAMP acts extracellularly as a chemoattractant and primary signal to control cell motility, aggregation (multicellularity), cytodifferentiation, pattern formation and cell type specific gene expression. We have



previously reported the isolation of four genes for distinct cAMP receptor subtypes, CARs 1, 2, 3 and 4. These genes have likely diverged from a common ancestor and comprise an entire cAMP receptor family. Each gene is expressed with distinct temporal and spatial patterns during the *Dictyostelium* developmental cycle.

We have extended our characterization of the mechanisms of control of transcription of two of these genes during *Dictyostelium* development. CAR1 has two promoters that are utilized at different developmental stages in response to different conditions of extracellular cAMP stimulation. The early CAR1 promoter is expressed during chemotaxis, induced by nM pulses of cAMP. Exposure of cells to non-fluctuating,  $\mu$ M concentrations of cAMP represses the early promoter and fully induces the late promoter. The two promoters generate mRNAs that differ only in their 5'-untranslated regions, encode identical proteins and appear to be enriched in prestalk cells relative to prespore cells. Early promoter sequences from -746 to +283 are sufficient for temporal, spatial and cAMP-regulated expression. Deletion of 6bp to -740 abolishes developmentally regulated expression of the early CAR1 promoter. Nuclei isolated from *Dictyostelium* during early development contain an *in vitro* DNA binding activity specific for sequences between -743 and -712. This activity is not detected in cells at later developmental stages. Mutation of four nucleotides within this sequence prevents both *in vitro* binding and *in vivo* expression of an otherwise wild-type, full-length promoter.

CAR3 mRNA is expressed maximally at 8-10 hr of development, the time prestalk and prespore cells are differentiating and forming loose aggregation mounds. The length of contiguous promoter sequences required for full activity is ~250bp, including upstream as well as transcribed sequences. Within this region we have defined two essential and distinct elements. One element lies near position -85 relative to transcription initiation and is unrelated to other promoter sequences described in *Dictyostelium*. The second element may be a member of the G-box/CACA family of regulatory sequences of *Dictyostelium*. Although there are multiple copies of this latter sequence within the CAR3 promoter, only one complete element is required for appropriate temporal or cAMP regulated expression of CAR3. Further, the single CACA element may be located either upstream or downstream of the transcription initiation site.

We have previously described the phenotypes of *Dictyostelium discoideum* bearing disruptions of the four different CAR genes. A more detailed investigation of the car4-genotype has been carried out with unique results involving differential expression of marker genes which are normally expressed in specific determined cell types. Cells having a CAR4 disruption progress through early development normally. At the time that CAR4 would be expressed, slug stage and early culmination, major aberrations in cell type specific gene expression and cellular patterning are observed. Markers for prestalk B cells are depressed whereas prespore markers are significantly overexpressed. Spatially, this is reflected in a reduction in B-cell type gene expression in the posterior and rear-guard regions of slugs of car4- nulls and detection of prespore gene expression in anterior and rear-guard regions, zones where normally only prestalk cells gene expression is observed. Further, prestalk A-cell type gene expression, primarily observed in anterior regions of parental slugs, is additionally detected in prespore regions of car4- null slugs. These patterning defects are



similarly manifested in mature fruiting bodies. These results extend the role for extracellular cAMP signalling to be throughout development and confirm a unique function for CAR4 in pattern formation and cytodifferentiation during late development.

In extension of our studies of development in *Dictyostelium discoideum* from *cis*-acting DNA elements to the *trans*-acting factors which regulate them, we have isolated a developmentally regulated cDNA that encodes a protein, zZIP1, containing leucine zipper and RING (zinc) finger motifs. The leucine zipper forms four helical repeats with incremental angles of  $\sim 100^\circ$ . Related proteins bind to DNA as homo- or heterodimers. The zinc fingers possess appropriately spaced cysteines and/or histidines that are members of the RING motif. They are predicted to form two closely linked domains that interact with  $\text{Zn}^{++}$ . The zZIP1 protein will likely dimerize through zipper interaction and bind nucleic acids via zinc fingers. zZIP1 also possesses glutamine, proline and charged regions that may be activator domains. zZIP1 is expressed maximally during development in multicellular structures (10-20 hrs). We have disrupted the zZIP1 gene by homologous recombination. These nulls aggregate poorly and form abnormal slugs with impaired migration. Relative to the levels of prestalk mRNAs, prespore marker genes are significantly overexpressed. Spatial patterning examined using cell type specific promoters fused to the *E. coli lacZ* gene shows aberrant prestalk regions in mutant slugs that are reduced in size compared to parentals. This is consistent with the absence of prestalk O cells and the relative underexpression of prestalk genes in the zzip1-background. Dissection of the role of zZIP1 protein in regulation of lineage specific genes will continue to be a major topic of investigation in *Dictyostelium* development.

Another *Dictyostelium* gene exhibiting both temporal and spatial expression pattern during development is SPIA. Expression of this gene is first detected at the apical region of the prespore mass during terminal differentiation of spore cells. As culmination proceeds, a gradient of expression becomes apparent. Precocious and enhanced expression of SPIA is observed in wild-type cells exposed to 8Br-cAMP or in prespore cells which overexpress the catalytic subunit of protein kinase A. Promoter analyses have localized a region required for both spatial and PKA regulation within  $\sim 100\text{bp}$ .

### **Lipid metabolism, lipases and genetic defects in both**

A connection between two major segments of the laboratory, those studying developmental biology at the molecular level and those interested in lipid metabolism, has been made in the past few years; the bridge was discovery of adipocyte specific, hormonally responsive proteins, perilipins, and cloning of their genes. Although the surface of the lipid storage droplet in adipocytes is the site of both deposition and retrieval of stored lipid, little if anything is known of the molecular details of reactions which occur at this critical juncture. Perilipins are associated with the surface of the lipid storage droplet.

The cDNA for one form of perilipin (perilipin A; 57 kDa) was cloned from a rat cDNA library. More recently, with probes based on the cDNA of perilipin A, we have found a second cDNA, termed perilipin B, which encodes a protein of 46 kDa. The A and B forms of perilipin have a common amino terminus, but differ in their carboxyl terminal regions. The predicted amino acid sequences for perilipins provide no basis for their resistance to

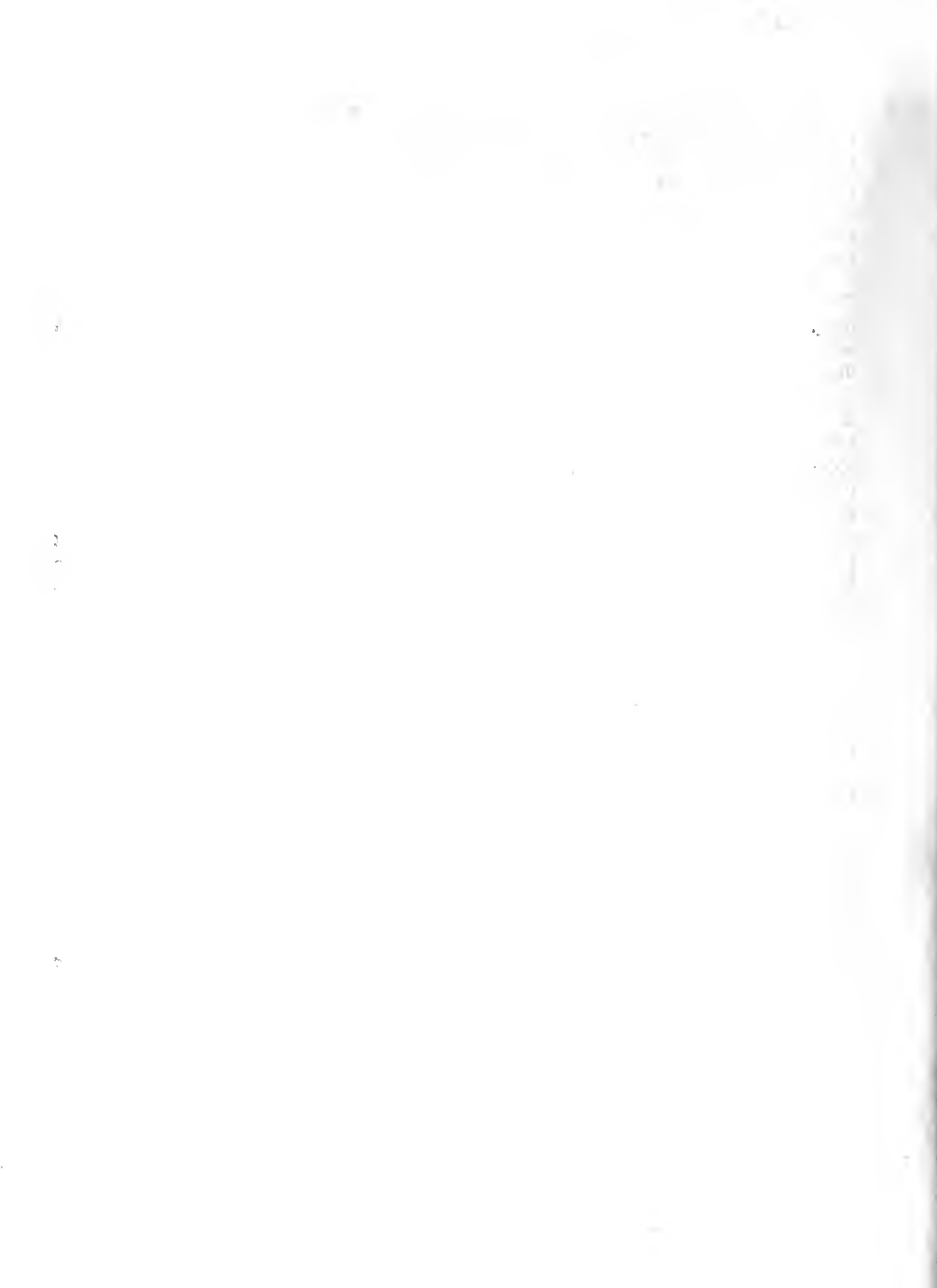


solubilization by a wide variety of chaotropic agents and detergents. A cDNA containing the full coding sequence for perilipin A was transcribed and translated in vitro with both the wheat germ and rabbit reticulocyte systems; the product migrated precisely with native perilipin in SDS-PAGE, suggesting that the behavior of perilipin in solution is not the result of post-translational modification. We have succeeded in solubilization and substantial purification of both perilipins A and B. The data indicate that perilipins A and B are the only major proteins unique to the lipid droplet.

The distribution of perilipin in differentiating 3T3-L1 adipocytes was studied. Perilipin and triacylglycerol containing lipid droplets appeared in developing cells simultaneously. Perilipin was present at the surface of both small and large lipid droplets in cells at all stages of development. Fluorescence microscopy showed also that endoplasmic reticulum, the intermediate filament vimentin, and peroxisomes all have close spatial relationships to developing lipid droplets in 3T3-L1 cells. Using electron microscopy, the triacylglycerol containing lipid droplet core was found to be surrounded by a surface layer in close apposition to tubules and cisternae of endoplasmic reticulum. Freeze-fracture studies show that the hydrophobic surface of the layer surrounding the lipid droplet core contains particles identical in size and structure to those found solely on the hydrophobic surfaces of leaflets of membrane bilayers and termed intramembranous particles (IMPs). The presence of IMPs indicates the surface layer of lipid droplets is derived from membrane leaflets, probably those of endoplasmic reticulum, the site of triacylglycerol synthesis. Electron microscopic immunogold studies show that perilipin is located on the IMP studded monolayer surface of the lipid droplet core.

To provide tools for examining perilipin function in cultured murine adipocytes, we have isolated perilipin cDNAs from a 3T3-L1 adipocyte library and have identified DNA sequences from a mouse genomic library. Full length coding sequences for genomic perilipin A and B reveal 93% identity with corresponding rat cDNAs, and the A and B forms contain different 5'-untranslated regions, indicating that the different forms of perilipin are regulated differently.

Although our earlier studies failed to reveal an obligatory relationship between lipid accumulation and perilipin expression, we now find that both biotin deprivation and the addition of avidin to cultured adipocytes lead to a commensurate reduction in both triacylglycerol and perilipin. Hormone-sensitive lipase (HSL), the rate limiting enzyme of lipolysis, is similarly reduced, whereas it is known that such conditions do not reduce the expression of lipogenic enzymes. These findings point to a role for perilipin in lipolysis, in line with its heavy phosphorylation under lipolytic conditions, a speculation reinforced further by studies with Y-1 adrenal tumor cell line. In adrenal cortical cells steroidogenesis is initiated by hydrolysis of stored cholesteryl esters by cholesteryl ester hydrolase, thought to be identical to HSL of adipocytes. Western blot analysis of subcellular fractions revealed that the Y-1 adrenal cells contain perilipins A and B. The fact that perilipin expression is apparently limited to cells in which lipid stores are marshaled by the action of HSL or a highly similar, if not identical, enzyme strongly suggests a role for perilipin in the hydrolysis of lipid by HSL or a related lipase.





Immunocytochemical studies were undertaken to extend this conclusion to other cells which synthesize and store triacylglycerol in the form of intracellular lipid droplets. We examined both lactating mammary gland and liver of newborn mice. In addition to adipocytes mammary gland contains alveolar epithelial cells which synthesize, store and secrete triacylglycerol in the form of milk lipid droplets during lactation. In mammary gland, perilipin was present at the surface of lipid droplets in adipocytes but not in alveolar epithelial cells. Hepatocytes of newborn mice, which store triacylglycerol in lipid droplets, also did not contain perilipin. Noted above, mouse Y-1 adrenal cells synthesize and store cholesterol ester and secrete steroids while SW 13 human adrenal cells accumulate cholesterol ester but lack steroidogenic capacity in culture. Perilipin was present at the surface of lipid droplets in Y-1 cells but not at the surface of lipid droplets in SW 13 cells. These findings are consistent with the conclusion that perilipin is present in cells which have the capacity to hydrolyze triacylglycerol or cholesterol ester via hormone sensitive lipase.

The critical reaction in hydrolysis of adipocyte triacylglycerols *in vivo* is translocation of the HSL to the lipid droplet surface. To reconstruct this event *in vitro*, lipid droplets from 3T3-L1 adipocytes were isolated under conditions where they retained their native morphology and had perilipin fully phosphorylatable by exogenous A-kinase. When droplets were incubated with cytosolic proteins, activation of A-kinase resulted in translocation of the cytosolic HSL to the droplets; this was blocked by a specific kinase inhibitor. If the *in vitro* translocation leads to lipid hydrolysis, we will have all the elements necessary to dissect the lipolytic process at the molecular level.

In addition to the data that link perilipin to lipid hydrolysis we have findings that point to a possible structural role. Chronic activation of cellular A-kinase and polyphosphorylation of perilipin results in an apparent dispersion of larger lipid droplets into numerous smaller droplets. We have produced 3T3-L1 cells containing a vector with perilipin cDNA in the antisense orientation; the cells contain no detectable perilipin and the lipid appears to be dispersed in microdroplets, reminiscent of the images seen in the chronically stimulated cells. Such data provide further evidence that perilipin is necessary for the maintenance of droplet structure or for the coalescence of smaller droplets to form larger droplets. Interestingly, cells transfected with perilipin in the sense orientation differentiate extremely rapidly; within 3-5 days these cells become filled with sizable lipid droplets, an image seen in normal cells only after 10-15 days. Thus, expression of both sense and antisense mRNAs has dramatic morphological consequences for the cultured adipocyte.

Two other proteins of importance in lipid metabolism have been studied in this laboratory for many years, lipoprotein lipase (LPL) and hepatic lipase (HL). Studies have been carried out in tissue culture and *in vivo* in both normal mice and mice with *cld/cld* combined lipase deficiency. Lipoprotein lipase is essential for utilization of plasma triacylglycerol (TG) by extrahepatic tissues. LPL is synthesized by parenchymal cells and acts at the luminal surface of capillaries where it is anchored by heparan sulfate proteoglycan. The active form of LPL purified from bovine milk is a noncovalent dimer of identical subunits with two N-linked oligosaccharide chains per subunit.



Cultured brown adipocytes synthesize and secrete active, dimeric LPL which has endo H-resistant, -partially resistant and -totally sensitive oligosaccharide chains. Secreted LPL normally has only endo H-resistant chains. This processing is not required; adipocytes treated with an inhibitor of Golgi mannosidase I (1-deoxymannojirimycin, DMM) secrete active, endo H-sensitive LPL. We reported earlier two conditions which resulted in synthesis of inactive, monomeric LPL which was retained in endoplasmic reticulum and had totally endo H-sensitive chains. These conditions were chemical inhibition of glucosidase I in endoplasmic reticulum (ER) (with castanospermine (CST)), which blocks removal of glucose residues from oligosaccharide chains, and combined lipase deficiency, a recessive mutation (*clt*) in mice which affects post-translational processing, not the structural gene, of LPL. Retention of LPL in ER and inactivity of LPL in CST-treated cells result from defective processing of oligosaccharide chains in ER. In *clt/clt* cells, it is not known whether the primary defect is faulty processing in ER or lack of transport of LPL from ER.

Brefeldin A (BFA) is known to block protein export from ER and cause dismantling of Golgi cisternae with relocation of Golgi enzymes into ER. We took advantage of the latter action of BFA to study the effect of Golgi enzymes on LPL retained in ER in glucosidase-inhibited and *clt/clt* adipocytes. LPL activity was increased from 10% to 85% of normal in CST-blocked brown adipocytes treated with BFA. About 85% of the LPL subunits were dimerized. BFA treatment also decreased  $M_r$  of LPL subunits, reflecting cleavage of glucose residues, and increased slightly the proportion of subunits processed to partial endo H-resistance. Similar but smaller effects occurred in *clt/clt* adipocytes treated with BFA. LPL activity was increased from 0% to 50% of normal in treated cells. About one-third of the LPL was dimerized. Most of the LPL subunits in BFA-treated *clt/clt* cells were processed to partial endo H-resistance, indicating that one chain of each subunit was processed to endo H-resistance. Processing of LPL to an active form requires the kinds of enzymes translocated to ER by BFA treatment. Whether these enzymes are located in Golgi cisternae or intermediate organelles is not known. Our findings in DMM-treated cells suggest that such enzymes, if involved in oligoglycan processing, would be located upstream from Golgi mannosidase I, possibly in the ER/cis-Golgi network. Because the sources of these enzymes are not known, the findings provide little information concerning the intracellular site of dimerization of LPL.

The data from BFA-treated *clt/clt* cells suggest that the inactive LPL in *clt/clt* cells probably results from impaired transport of LPL from ER, a conclusion previously made on the basis of immunocytochemical studies, wherein hepatocytes from *clt/clt* mice displayed LPL in a reticular pattern characteristic of ER and in lysosomes. In contrast, in BFA-treated cells, LPL is only in ER and not lysosomes, suggesting a degradation pathway for the unprocessed enzyme in the mutant mice. The fact that other proteins are not affected suggests that the transport defect is LPL-specific. Others have suggested that LPL may be anchored to intracellular membranes by heparan sulfate proteoglycan, or glycosyl phosphatidylinositol, or a combination of both. The transport defect in *clt/clt* cells may involve a defective LPL-anchoring system, or a structural change in the LPL molecule preventing interaction with the anchor.

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Mice born with combined lipase deficiency (*clد/clد*) have very low lipoprotein and hepatic lipase activities, develop severe hyperchylomicronemia, and die within 3 days after birth. We recently initiated a study to determine the effect of the *clد* mutation on chylomicrons and other plasma lipoproteins. Plasma lipoproteins were separated by differential ultracentrifugation into four groups: chylomicrons, very low density lipoproteins (VLDL), intermediate and low density lipoproteins (IDL+LDL), and high density lipoproteins (HDL). Apoproteins were determined in each class.

Plasma triglyceride concentration was 4,900 mg/dl in *clد/clد* mice, about 250 times that in newborn normal mice. The ratio of cholesteryl ester to cholesterol was <1/5 of that in normal lipoproteins, indicating an impairment of cholesterol esterification. The major apoproteins in *clد/clد* chylomicrons and lipoproteins differed in detail from the distribution observed for normal mice. Apo B-48 is normally synthesized in intestines and apo B-100 in liver. Both apoproteins are prominent components of IDL+LDL in normal mice, but not in *clد/clد* mice. It appears that synthesis of apo B-100 may be seriously impaired in liver of *clد/clد* mice. Our earlier electron microscopic studies showed that lipoprotein particles, probably newly synthesized VLDL, in ER and Golgi of liver were much fewer and much smaller in *clد/clد* than in newborn normal mice. It is of interest that apo B is a glycoprotein with N-linked oligosaccharide chains. The possibility that the *clد* mutation may impair glycosylation of apo B-100 will be studied further.

Transfer of apo B-48, apo A-IV and apo E from chylomicrons to other lipoproteins is virtually absent and transfer of apo A-I is reduced 50% in *clد/clد* mice. Movement of these apoproteins to other lipoproteins is dependent, in part, on extensive hydrolysis of chylomicron triglyceride by LPL; it is likely that LPL deficiency in *clد/clد* mice accounts for some of these changes. Normal cholesterol esterification in blood may require release of one or more of the apoproteins retained in *clد/clد* chylomicrons.

We have previously reported studies of the movement of cholesterol in normal cells and those from patients with Niemann-Pick Type C disease. The critical role of the Golgi in transport was suggested in these studies. Various compartments appeared to mediate movement of cholesterol derived from exogenous LDL from endocytotic vesicles to lysosomes to ER for esterification. We have now established a role for the intermediate filament protein, vimentin, in lysosomal mobility and cholesterol esterification. Mutant human adrenal tumor cells that lack vimentin have an impaired ability to esterify cholesterol. Immunocytochemical studies of cells with and without vimentin show different distributions of lysosomes and the vimentin network. Without vimentin, lysosomes are clustered near the nucleus in contrast to the wild type dispersed morphology for these organelles. Expression of a vimentin cDNA in mutant cells restores esterification and redistribution of lysosomes to radial positions. In contrast to the role of Golgi in cholesterol trafficking suggested by studies of fibroblasts, these results suggest a direct lysosomal transfer pathway for cholesterol to ER for esterification in some cell types.

The effects of cytokines on lipases and other proteins has been suggested to be important in the cachexia of wasting illnesses. Previous studies in this laboratory have shown that both IL-6 and TNF inhibit adipogenesis and lipoprotein lipase activity in 3T3-L1 cells, and that



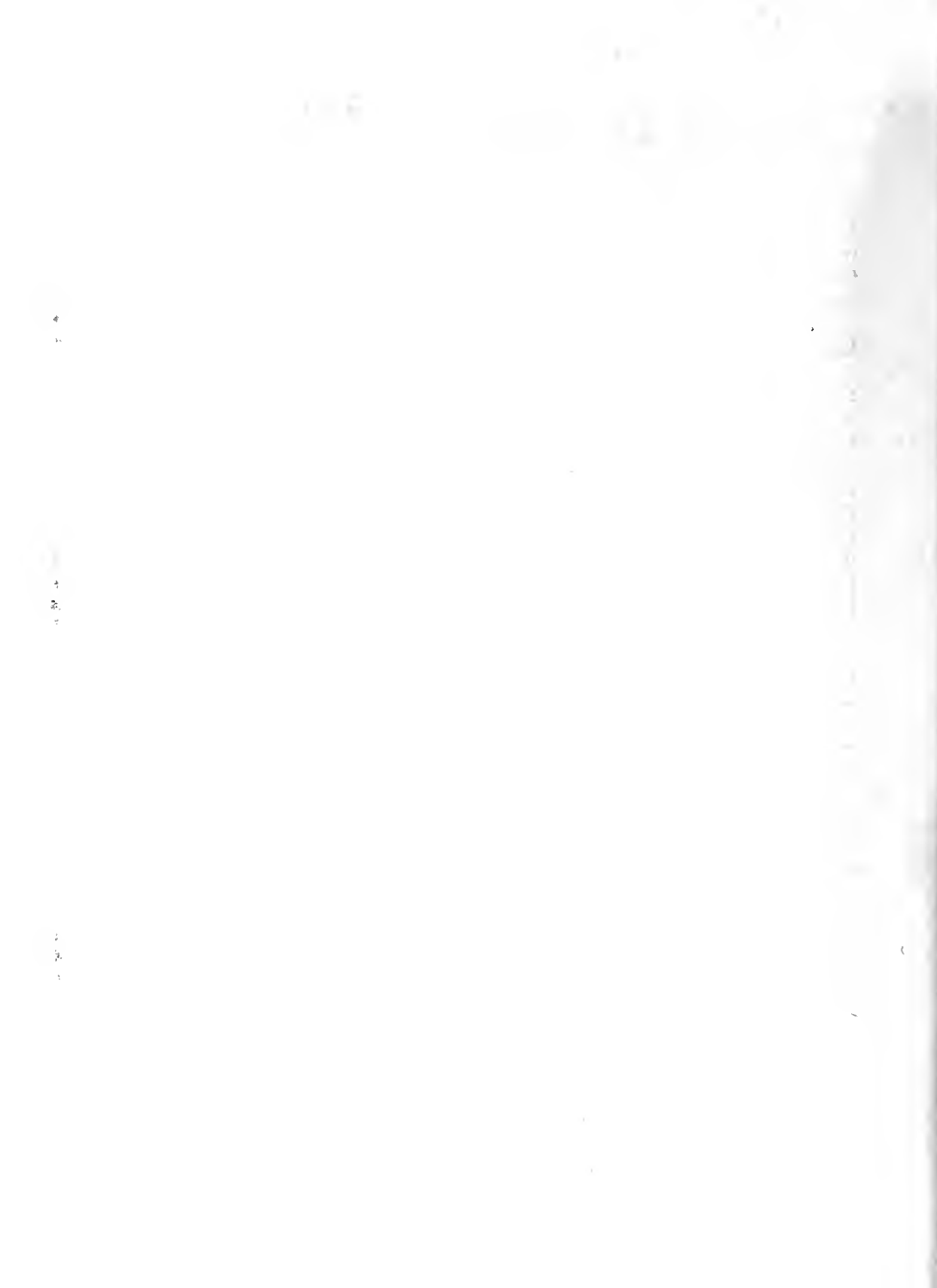
TNF also induces the production of IL-6. Phosphorylation on tyrosyl residues of proteins of apparent mass 44, 84, 92/94 and 130 kD was observed in cultured adipocytes treated with IL-6. Only p44 was phosphorylated in cells treated with TNF. This protein has been identified as MAP kinase using immunoprecipitation with anti-MAP kinase antibody. MAP kinase activity is increased in adipocytes treated with IL-6. These results suggest that TNF and IL-6 mediate their actions by different signalling pathways and that changes in protein tyrosine phosphorylations may be important in the actions of IL-6. A possible overlap site for the actions of the two cytokines, perhaps important in their common effects on gene expression, is phosphorylation and activation of MAP kinase.

### **Enzymology and protein structure**

Several proteins of high interest in their own right or as models for fundamental processes in biochemistry are studied in the laboratory. Dihydrofolate reductase (DHFR) is the target enzyme for antifolate drugs which are widely used in treatment of neoplastic and autoimmune diseases. In addition to dihydrofolate, the reduction of other pteridines with aliphatic substituents at C-6 is also catalyzed by DHFR although with decreased efficiency. Of particular biological interest in this regard is the reduction of dihydrobiopterin, cofactor for the monooxygenases that hydroxylate tyrosine, phenylalanine and tryptophan. Although the enzyme that specifically reduces biopterin derivatives is dihydropteridine reductase, DHFR has been shown to function in a salvage pathway. We have shown that vertebrate DHFR can reduce dihydrobiopterin at 3% (beef liver DHFR) to 50% (chicken liver DHFR) of the rate of dihydrofolate reduction.

In collaboration with others, the 2.0 Å crystal structure of chicken liver DHFR in a complex with NADP and biopterin has been determined; this is the first ternary complex structure of an animal DHFR with both a substrate and cofactor. Analysis of the higher resolution structure has shed further light on a possible mechanism of substrate protonation and on the possible catalytic function of the p-aminobenzoylglutamate (PABG) moiety of folate substrates. This analysis demonstrates that the reduction of dihydrofolate and dihydrobiopterin occur by similar mechanisms for chicken liver DHFR. In contrast, the mechanism for reduction of dihydrobiopterin by dihydropteridine reductase appears to be quite different.

An important series of steps in infection by HIV-1 are the proteolytic cleavage of the polyprotein translated from the polycistronic mRNA by HIV-1 protease (PR). A new series of investigations in LCDB addresses aspects of the activation, dimerization, mechanism and inhibition of this important enzyme. Like other aspartic acid proteases, retroviral PRs possess an active site containing two sequences of AspThrGly. Cellular aspartic acid proteases are monomers, but for HIV-1 PR a homodimer formation is required to form the active site. This feature clearly distinguishes the HIV PR from the cellular aspartic acid PRs, and can be used to design specific inhibitors of dimerization. The initial step in polyprotein processing is presumed to be the folding and dimerization of the Gag-Pol polyprotein to form the first active PR. Such polyprotein dimers can then be responsible for the release of the "active form" of the PR necessary for the processing of the Gag and the Gag-Pol polyproteins to produce the necessary functional proteins for viral infectivity. In order to





attempt studies of the inhibition of the PR at the polypeptide level, we have developed an expression system for the production of inactive polypeptide mimics of the PR primarily to study the detailed mechanism of the activation process.

The protease coding region of the human immunodeficiency virus, type 1 (HIV-1) with short flanking region sequences was expressed in fusion with the maltose-binding protein of the *malE* gene of *Escherichia coli*. The fusion protein [MBP-12AA-PR-19AA (52 kDa)] was isolated by affinity chromatography and purified to near homogeneity by size-exclusion column chromatography. The fragmentation of the MBP-12AA-PR-19AA was analyzed by SDS-PAGE and immunoblotting. A spectrophotometric assay was employed to follow specific enzymatic activity. Based on the facts that the autoprocessing reaction (i) is a first-order process, (ii) is inhibited by pepstatin A and synthetic peptide substrates of the mature PR, and (iii) requires a catalytic group with an apparent  $pK_a$  of 6.7 in its protonated form, we are proposing the following mechanism for autoprocessing of the HIV-1 PR. The denatured MBP-12AA-Protease-19AA renatures, folds and dimerizes to form a structure which we term Tetrapod (dimeric protease containing uncleaved N and C-terminal strands). The competitive inhibition of the autoprocessing reaction by pepstatin A requires the presence of the two forms of the tetrapod. Tetrapod-I has low enzymatic activity relative to the mature enzyme and has a binding site that is similar but not necessarily identical to that of the mature enzyme and accessible to bind substrates and inhibitors. It is in equilibrium with its conformational isomer tetrapod-II. Tetrapod-II, an obligatory reaction intermediate on the reaction pathway, has one of its N-terminal strands bound to its own active site which corresponds to the Michaelis-complex in regular enzyme-catalyzed reaction. The similarity between the catalytic machinery of the active sites of the mature protease and tetrapod-I is confirmed by the pH rate profile for autoprocessing. The observation of first-order kinetics for the N-terminal cleavage for more than three half-lives in the presence of pepstatin A (0 to 1  $\mu M$ ;  $K_i = 0.2 \mu M$ ) is consistent with a rapid equilibrium between the two forms of the tetrapod relative to the step that commits the tetrapod for the hydrolytic reaction. Cleavage of the N-terminal strands of tetrapod-I is a rate-limiting step for the disappearance of MBP-12AA-PR-19AA and appearances of the product MBP-12AA and enzymatic activity. The tripod (dimeric MBP-12AA-PR-19AA containing only one uncleaved N-terminal strand) does not accumulate in the reaction beyond a steady-state concentration and is converted to the bipod (PR-19AA) in a fast step. The bipod possesses enzymatic activity similar to that of the mature PR. The bipod is then converted to the mature PR. Molecular modeling studies in collaboration with K. Parris, LMB, NIDDK, show that it is possible to fit the N-terminal 12AA strand into the active site cleft of HIV-1 protease without causing much distortion in the 3-D structure of the mature enzyme. Furthermore, comparing the mechanism of conversion of pepsinogen to pepsin, a mammalian aspartic acid protease, to that of tetrapod-I to the bipod reveals several striking similarities. Thus, it appears that the cleavage of an N-terminal peptide bond to activate aspartic acid proteases from their relatively inactive precursors is a common feature that is maintained throughout the evolutionary process.

In order to further define the mechanism of activation of HIV protease, we have begun several different lines of experimentation, including crystallization of the protease fusion



protein with pepstatin A for structural analysis and purification of the 13.2 kD intermediate. Further studies of the role of protease in the virus life cycle include identification of a predicted protease cleavage site within the nucleocapsid precursor protein of HIV and determination of the kinetic parameters for its cleavage, using a synthetic polypeptide.

Characterization of the HIV-1 PR has been facilitated by a collaborative study with the Biotechnology Unit of LCDB; the large scale culture and purification scheme developed in the laboratory enables isolation of pure PR that is several orders of magnitude more active than that from any currently available commercial source.

Barnase, an extracellular ribonuclease of *B. amyloliquefaciens*, and barstar, its intracellular inhibitor, are both small proteins which form a one to one complex and undergo two state physical transitions. Barnase has become the system of choice for protein folding experiments; over sixty articles using barnase as the experimental system for study of protein folding mechanisms as well as other uses have been published in the past three years. The barnase/barstar complex seems likely to become the paradigm for study of protein protein interactions in the same fashion that barnase is for folding. Both genes have been cloned, sequenced, expressed and subjected to directed mutagenesis.

The structures of the barnase-barstar A complex (barstar A is the double mutant (C42,80A)) and wild-type barstar alone have been solved and refined in Dr. Yves Mauguén's laboratory. Each was done with data from a single crystal using molecular replacement techniques, starting with the known barnase structure. The structure of the complex has confirmed our identification of four residues, H102 and R59 of barnase and D35 and D39 of barstar, as being directly involved in the interaction. The structure has, in fact, identified some 10 residues of barnase and 14 of barstar as being in direct contact in the complex. We have now prepared one or more mutants of each of the 14 contact residues of barstar and done a preliminary screen of the effects of most of these mutations on the free energy of binding by running barnase-barstar titration curves. This technique, with our current assay sensitivity, can measure dissociation constants in the range from  $ca. 10^{-11}$  M to  $10^{-8}$  M ( $\Delta G$  values of 14 KCal to 9 KCal). For the wild-type pair the value is  $10^{-13}$  M (18 KCal). Barstar mutations which reduced binding by  $10^2$  to  $10^5$  were Y29A, Y29P, G31A, N33A, D35A and D39A. The barstar mutants Y29F, Y30W, L34A, W38Y, W38F, T42A, T42S, G43A, and E46A all bind barnase too tightly for their dissociation to be measured by titration, but will be amenable to our more tedious barnase competition assay. The mutants Y30A, Y30F and A36G yield barely detectable activity.

We have used our *in vivo* system to select barstar mutants which inhibit barnase(H102K) better than does the wild-type. The efficacy of such a mutant in suppressing the lethal effect of this barnase mutant can be judged by the amount of IPTG inducer of the barnase gene necessary to overcome the suppression. The barstar mutations Y29P and Y30W are such barstar mutants, with the latter being the more effective. A double mutant Y29P,Y30W is somewhat better yet. The double mutant Y29I,Y30G, selected together in a single experiment, also suppresses, but to a lesser degree than the others. It seems likely that these mutations are exerting their effect through substantial modifications of the barstar structure. The phenolic side chain of Y30 is completely buried in barstar and contacts barnase only



through a hydrogen bond between its main-chain carbonyl group and H102 of barnase. This carbonyl group is flipped 180° in the wild type complex relative to its position in free barstar. The barstar mutant E76R, on the other hand, suppresses the damaging effect of barnase(R59E) by simply restoring the charge interaction in the opposite direction.

We previously suggested that this system could be used for direct selection of secondary mutations in either barnase or barstar that compensate primary mutations which interfere with inhibition. We further thought that we could thus explore the interface between the two proteins as well as asking direct questions about protein protein interactions by finding compensatory mutants that suppress mutations that disrupted barnase barstar complex formation. This proposal has proven true.

Protein-protein interactions are also important in the species specific recognition of oocyte by sperm. Mouse ZP3 and ZP2 have been reported to act as primary and secondary sperm receptors, respectively, and O-linked oligosaccharides of ZP3 have been implicated in initial sperm-zona interactions. We have established conditions for mouse *in vitro* fertilization and will use competition by recombinant mouse zona proteins, mutations of same and chimaeric human-mouse zona proteins to define domains important for species specific events in fertilization. Currently, this project is at the stage of expressing and purifying sufficient amounts of the four wild type proteins to begin the biochemical analyses. The cDNA clones with a histidine tag in a plasmid based expression vector with a selectable marker are in hand. In the case of mouse ZP3, expression in CHO cells following electroporation of the plasmid has been shown by Western blot.

We have continued to distribute the model substrates for higher order chromatin structure that contain tandemly repeated nucleosome positioning DNA sequences to other laboratories; these molecules are currently in use in nearly twenty other sites. Dr. Timothy Richmond at the ETH in Zurich, Switzerland, has nearly completed the determination of a high resolution X-ray crystal structure of the core particle of the nucleosome, using a unique DNA fragment based on the 5S rRNA positioning signal and constructed in this laboratory in a collaborative effort.

## **Biotechnology**

Certain aspects of research done in the laboratory have actual or potential applications in the clinic or field. Barnase and barstar have been used by others to create male sterile plants and to reverse the sterility by expression of the inhibitor. This advance should have a major impact on the crop seed business; it may well be the most significant application of genetic engineering to agriculture thus far.

The Biotechnology Unit of the laboratory is a research and development facility in addition to providing fermentation and processing services for all of the NIH community. During the past year, the unit performed 252 large scale preparations including growth of microorganisms in volumes up to 300 liters and eukaryotic cells in volumes up to 50 liters. The Unit continues its activity to produce exotoxins for use in chimaeric therapeutic drugs; this year, recombinant *Pseudomonas aeruginosa* exotoxin A that lacks the binding activity but

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retains the enzymatic activity was a major product. After development of a suitable large-scale production and purification procedure, 10 gr of purified, suitable for clinical trials, protein were produced. The final product has a low content of endotoxin and residual DNA. The Unit continues the production of S antigen from bovine retina which is currently in first and second phase clinical trials as a potential treatment for autoimmune diseases.

This overview of the work of the Laboratory of Cellular and Developmental Biology is a brief summary of the studies carried out in the past year by talented and productive scientists. We hope that the connectiveness of our science and its diversity leads the reader to a sense of the excitement we feel in our daily interactions as we move forward in understanding of the most interesting aspects of cellular and molecular biology in a very exciting time for modern biology.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 DK 15100-23 LCDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Protein Nucleic Acid Interactions: Chromatin Structure and Function**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	R.T. Simpson	Laboratory Chief	LCDB:NIDDK
Others:	Julia P. Cooper	Staff Fellow	LCDB:NIDDK
	Michael P. Kladde	Staff Fellow	LCDB:NIDDK
	Randall H. Morse	Senior Staff Fellow	LCDB:NIDDK
	Michael Murphy	IRTA Fellow	LCDB:NIDDK
	Hugh-George Patterson	Visiting Fellow	LCDB:NIDDK
	Christopher Szent-Gyorgyi	Senior Staff Fellow	LCDB:NIDDK

COOPERATING UNITS (if any)

T. Richmond, ETH, Zurich, Switzerland (foreign)

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

6.5

PROFESSIONAL:

6.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unenriched type. Do not exceed the space provided.)

The role of chromatin structure in modulating the functions of DNA in transcription, replication, recombination and repair is becoming increasingly apparent. We have shown that the yeast alpha2 repressor positions a nucleosome adjacent to its operator. This organized chromatin structure is propagated; at least three additional nucleosomes are positioned with base pair precision. This structure seems to be the mechanism of repression; mutations in several yeast genes whose function is known to be necessary for repression by alpha2 also lead to perturbation of the organized chromatin structure. Studies moving the transcription initiation site relative to the alpha2 operator demonstrate that repression is involved with chromatin structure above the level of an individual nucleosome. In the case of one haploid specific gene, an organized chromatin structure is found near an alpha1/alpha2 binding site in diploids. Hierarchies exist in the contest between transcription factors and histones. While alpha2 can organize chromatin structure and repress transcription of a-cell specific genes, the GAL4 protein can bind to its operator, disrupting formation of a stable nucleosome. A systematic study of the use of prokaryotic dam methylase for probing chromatin structure has defined the accessibility of nucleosomal, linker, and hypersensitive region DNA to the modifying protein. Studies of the expression of the sporulation induced HSP82 gene have defined elements that are necessary for normal expression, although likely not for meiosis responsiveness, and an apparent repressor sequence. Collaborative studies of higher order chromatin structure and the crystal structure of a nucleosome core particle containing unique sequence DNA continue.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 15102-33

## PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of a ribonuclease and its inhibitor from *Bacillus amyloliquefaciens*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Robert W. Hartley	Research Physicist	LCDB:NIDDK
	Milan Jucovic	Visiting Fellow	LCDB:NIDDK
	Yelena Chernokalskaya	Visiting Associate	LCDB:NIDDK

COOPERATING UNITS (if any) Dr. Yves Mauguen, Lab de Physique, Centre Pharmaceutique, Univ de Paris-Sud. Dr. Josef Sevcik, Inst of Molecular Biology, Slovak Acad Sci, Bratislava. Dr. Marat Karpeisky, Inst of Molecular Biology, Russian Acad Sci, Moscow. Dr. Alexander Makarov, Inst of Molecular Biology, Russian Acad Sci, Moscow. Dr Franklyn Prendergast, Mayo Foundation, Rochester, Minn.

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Developmental Chemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL STAFF YEARS:

3

## PROFESSIONAL:

3

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

Two proteins, barnase, the extracellular ribonuclease of *Bacillus amyloliquefaciens*, and barstar, its intracellular inhibitor, are used as a model system for the study of protein folding and protein-protein interactions. Barnase is one of an homologous group of ribonucleases occurring in both prokaryotes and eukaryotes.

Recombinant DNA techniques are being applied with three major aims: (1) to facilitate production of wild type and mutant proteins; (2) to examine the structural and control sequences of the genes; and (3) to make specific changes in the sequences to test theories of folding and to probe the barnase-barstar interaction.

Both proteins can now be obtained from recombinant genes in *E. coli* with yields of 100 mg/l or better. Co-expression of barstar is necessary to counter the lethal effect of barnase expression. X-ray structures of both proteins and their complex are known as well as the NMR solution structure of barnase. A fast and relatively precise assay based on a fluorogenic substrate has allowed us to use barnase-barstar titration curves and competition between active and inactive barnase mutants for barstar to study the kinetics and stability of complex formation. Solution of the structure of the complex has confirmed the involvement of several residues identified as such by protein engineering. One or more mutations of all of the barstar residues in direct contact with barnase in the complex, as well as several such for barnase, have been prepared. Determination of the Gibbs free energy for all combinations of such mutants in complex will isolate the energy contributions of different portions of the interface. Barstar A (barstar(Cys42,80Ala)), Which binds barnase almost as well as the wild type, is being used for much of this work. A system has been devised to select, *in vivo*, barstar mutants with improved binding to barnase mutants which are poorly inhibited by wild type barstar. Several such barstar mutants have been found.

Recent work, elsewhere, in which the barnase gene is attached to a eukaryotic promoter in order to kill the tissue in which the promoter is expressed (in the first instance to produce male sterility in plants) has aroused considerable interest in its use in developmental studies and is the key to a variety of anti-viral strategies.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 DK 15200-33 LCDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on Folic Acid (Dihydrofolate Reductase) and Vitamin A (Beta-Carotene)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Bernard T. Kaufman	Section Chief	LCDB:NIDDK
Others:	John G. Bieri	Scientist Emeritus	LCDB:NIDDK

COOPERATING UNITS (if any)

Dr. J.C. Smith, U.S. Department of Agriculture, Beltsville, MD; Dr. James Freisheim, Medical College of Ohio; Dr. Michele McTigue, University of California at San Diego

LAB/BRANCH

Laboratory of Cellular & Developmental Biology

SECTION

Nutritional Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2

PROFESSIONAL:

1

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

This project continues to focus on dihydrofolate reductase, a critical enzyme in the metabolism of the B-vitamin folic acid (a pteridine), and beta-carotene, a precursor of vitamin A. The maintenance of folic acid at the fully reduced level, tetrahydrofolate, by dihydrofolate reductase is essential to cellular survival. Anti-folate drugs which target this enzyme continue to be widely used in cancer treatment and an increasing number of autoimmune diseases. Beta-carotene is currently receiving considerable attention as a biological antioxidant, particularly with respect to cardiovascular disease.

The enzymatic reduction of other pteridines is also catalyzed by dihydrofolate reductase, although with decreased efficiency. Of particular biological interest is the reduction of biopterin, the cofactor for the enzymes that hydroxylate tyrosine, phenylalanine and tryptophan. The enzyme that specifically reduces biopterin is pteridine reductase. However, we have shown that dihydrofolate reductase can reduce dihydrobiopterin from 3% in beef liver to 50% in chicken liver, as compared with the rate of dihydrofolate reduction. Furthermore, biopterin is tightly bound to dihydrofolate reductase. The chicken liver dihydrofolate reductase has been crystallized not only as a binary and ternary complex with substrates and inhibitors, we now report the crystallization and x-ray analysis of a complex with biopterin and NADP, a ternary complex with both substrate and cofactor. X-ray analysis of this structure has shed further light on a possible mechanism of substrate protonation and on the possible catalytic function of the p-aminobenzoylglutamate moiety of folate substrates.

In continuing studies of carotene metabolism, the effect of copper deficiency on the conversion of beta-carotene to vitamin A (retinol) was measured. The rationale was that the enzyme postulated to convert carotene to retinol in the intestinal wall is a dioxygenase containing copper. Copper-deficient rats were found to store retinol as efficiently as copper-adequate rats. When beta-carotene was fed to copper-deficient rats, liver storage of retinol, a measure of carotene absorption and conversion, was not different from controls. These results bring into question the possible role of a dioxygenase in carotene conversion to retinol.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

201 DK 15401-21 LCDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthesis and Transport of Lipoprotein and Hepatic Lipases in Cells and Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Robert O. Scow	Chief, Endocrinology Section	LCDB:NIDDK
Others:	Charles J. Schultz	IRTA Predoctoral Fellow	LCDB:NIDDK
	E. Joan Blanchette-Mackie	Research Biologist	LCDB:NIDDK
	Albert E. Spaeth	Chemist	LCDB:NIDDK
	Do-Gon Ryu	Special Volunteer	LCDB:NIDDK

COOPERATING UNITS (if any)

Dr. Jin-Woo Park, Department of Biochemistry, Chonbuk National University Medical School, Chonju, Chonbuk, Republic of Korea; Dr. Tsuneo Takahashi, Department of Oral Anatomy, Kanagawa Dental College, Yokosuka, Kanagawa, Japan

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Endocrinology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.00

PROFESSIONAL:

1.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

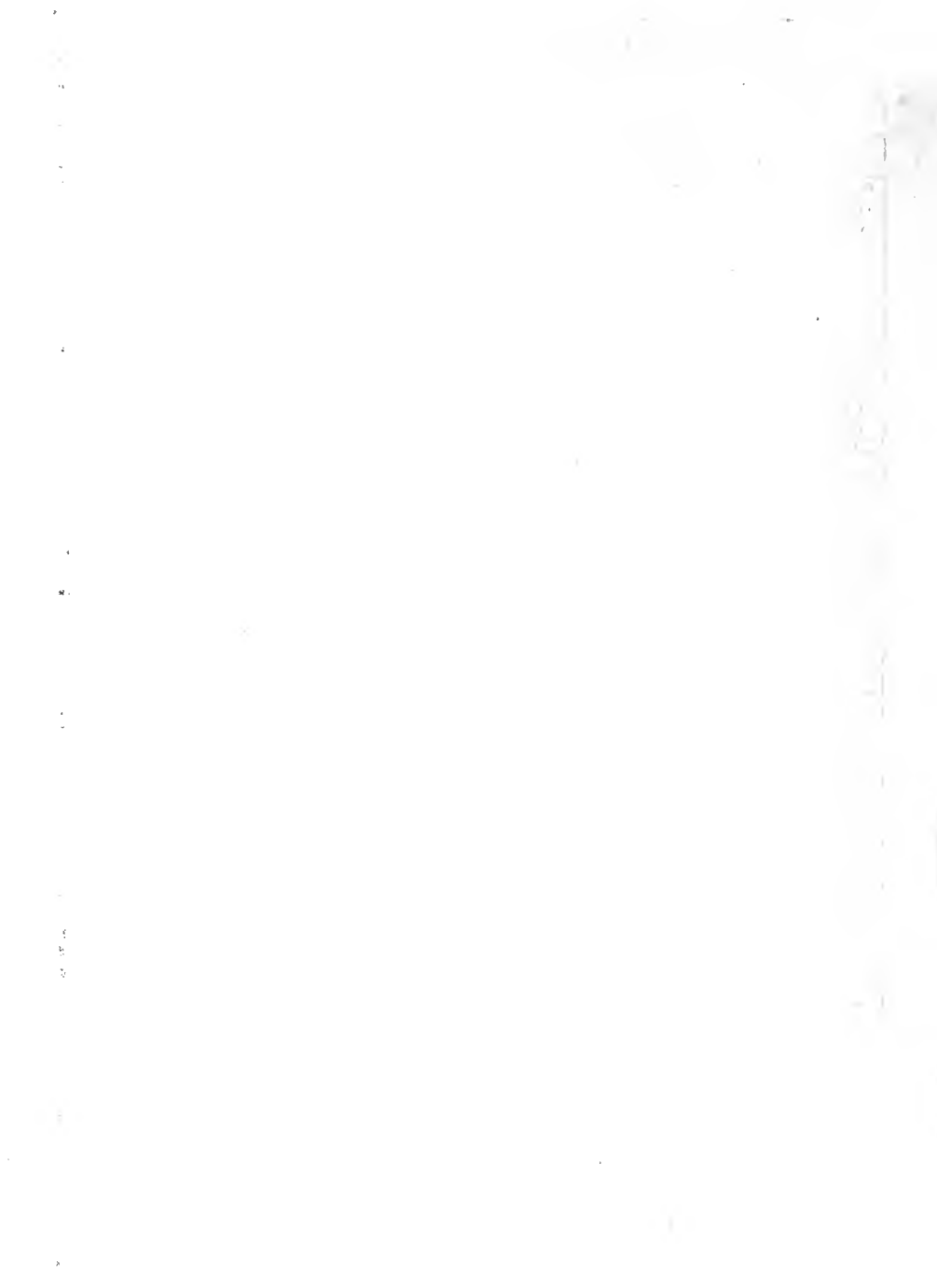
- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Lipoprotein lipase (LPL), which is essential for utilization of plasma triacylglycerol (TG), is synthesized by parenchymal cells and acts in capillaries where it is anchored by heparan sulfate proteoglycan. Brown adipocytes cultured from newborn mice synthesize LPL which is active, dimerized ( $M(r) = 110,000$ ) and secreted, and has endo H-resistant, -partially resistant and -totally sensitive oligosaccharide chains, and high affinity for heparin-Sepharose (eluted with 0.9 M NaCl).

Combined lipase deficiency (*clld*) is a recessive mutation in mice which affects post-translational processing, not the structural gene, of LPL. Cultured *clld/clld* brown adipocytes synthesize LPL which is inactive, not dimerized, and retained in endoplasmic reticulum, and has totally endo H-sensitive chains and low affinity for heparin-Sepharose (eluted with 0.45 M NaCl). It is not known whether the primary defect in *clld/clld* cells is faulty processing in ER or lack of transport of LPL from ER. Brefeldin A (BFA), which causes relocation of Golgi enzymes into ER, was used to study the effect of Golgi enzymes on LPL retained in ER. LPL activity was increased from 0% to 50% of normal in *clld/clld* cells treated 4 h with BFA. About one-third of the LPL was dimerized, and one-tenth acquired high heparin-affinity. Most of the LPL subunits in BFA-treated *clld/clld* cells were processed to partial endo H-resistance. The active LPL in BFA-treated *clld/clld* cells was dimeric and had high affinity for heparin. The findings indicate that synthesis of inactive LPL in *clld/clld* cells probably results from impaired transport of LPL from ER. The transport defect may involve a defective LPL-anchoring system, involving heparan sulfate proteoglycan or glycosyl phosphatidylinositol, or a structural change in the LPL molecule preventing interaction with the anchor.

Plasma of *clld/clld* mice has 250 times normal triacylglycerol concentration and one-tenth normal ratio of cholesteryl ester to cholesterol. Most apo E and apo A-IV in plasma of *clld/clld* mice were present in chylomicrons, whereas apo E in newborn normals was mostly in HDL, and apo A-IV was in VLDL and HDL. Apo A-I in *clld/clld* mice was equally distributed between chylomicrons and HDL, whereas all apo A-I in normal mice was in HDL. Normal cholesteryl esterification may require release of one or more of the apoproteins retained in *clld/clld* chylomicrons. Preliminary findings suggest synthesis of apo B-100, a glycoprotein with N-linked oligosaccharides, is impaired in *clld/clld* mice.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15404-09 LCDB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

Ultrastructural Immunocytochemistry of Lipid Metabolism in Cells and Tissue

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	E. Joan Blanchette-Mackie	Research Biologist	LCDB, NIDDK
Others:	Robert O. Scow	Chief, Endocrinology Sect.	LCDB, NIDDK
	Nancy K. Dwyer	Biologist	LCDB, NIDDK
	Robert A. Coxey	IRTA, Post Doctoral Fellow	LCDB, NIDDK
	Therese Barber	IRTA, Pre-Doctoral Fellow	LCDB, NIDDK

## COOPERATING UNITS (if any)

Dr. Peter Pentchev, Dev Metab. Neurol Branch, NINCDS, NIH; Dr. Howard Kruth Lab Exptl Ather, NHLBI, NIH; Dr. Constantine Londos, Lab Cell Develop Biol, NIDDK, NIH; Dr. Robert M. Evans, Dept. of Pathology, U. Colorado, CO.

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Endocrinology Section

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda Maryland 20892

## TOTAL STAFF YEARS:

3.5

## PROFESSIONAL:

2.0

## OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☒ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

Intracellular location and transport of cholesterol was studied in normal cells and those with genetic defects in lipid metabolism by fluorescence and electron microscopy. Golgi was identified as regulatory organelle for cholesterol transport. In normal fibroblasts both trans Golgi vacuoles and cis/medial Golgi cisternae accumulate cholesterol in response to LDL uptake suggesting a route of transport for cholesterol from trans Golgi to plasma membrane and cis/medial Golgi to endoplasmic reticulum (ER). In NP-C fibroblasts defective in cholesterol metabolism, cholesterol accumulates in trans Golgi cisternae suggesting impaired cholesterol transport through Golgi. Lipid metabolites of ceramide formed in Golgi, are also affected by cholesterol enrichment of Golgi membranes. Ability of cells to process lipids and lipoproteins could depend on modulation of cholesterol enriched membrane traffic through Golgi. Studies on human adrenal cells indicate a direct lysosomal transfer pathway may transport LDL cholesterol to ER for esterification. Perilipin is present at the surface of intracellular lipid droplets. ER, vimentin and peroxisomes have a close spatial relationship to developing lipid droplets in 3T3-L1 adipocytes. Electron microscopy showed that particles associated with surface layer of lipid droplets are identical to IMP of membrane bilayers, indicating that the lipid droplet surface is derived from a membrane leaflet, probably ER where triacylglycerol is synthesized. Perilipin is located on this monolayered surface of the lipid droplet core. Perilipin was also located on surface of cholesterol ester enriched lipid droplets in Y1 mouse adrenal cells which synthesize steroids upon hormonal stimulation. Thus, perilipin is present in cells which have capacity to hydrolyze triacylglycerol or cholesterol ester via hormone sensitive lipase. We located lipoprotein lipase (LPL) and hepatic lipase (HL) in livers and cultured hepatocytes from newborn mice. In contrast to hepatocytes from normal mice those from cl/d/cl/d mice retain LPL intracellularly in ER and lysosomes. Liver and hepatocyte cultures from cl/d/cl/d mice contain HL in the extracellular environment. Studies with monensin indicate that HL is transported to Golgi in both normal and cl/d/cl/d hepatocytes. Thus cl/d/cl/d hepatocytes are capable of transporting HL but not LPL from ER to Golgi for processing and secretion. Cultured cl/d/cl/d brown adipocytes retain LPL in ER although studies indicate Golgi enzymes necessary for processing of LPL are present and active in these cells. Findings in brown adipocytes and hepatocytes indicate a primary transport defect specific for LPL in cl/d/cl/d cells.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 DK 15500-32 LCDB

**PERIOD COVERED**

October 1, 1992 to September 30, 1993

**TITLE OF PROJECT** (80 characters or less. Title must fit on one line between the borders.)

Large-Scale Production and Purification of Compounds with Biological Activity

**PRINCIPAL INVESTIGATOR** (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Joseph Shiloach	Research Chemist	LCDB:NIDDK
Others:	Jeanne B. Kaufman	Biologist	LCDB:NIDDK
	Amos M. Tsai	Pre-IRTA Fellow	LCDB:NIDDK
	Susan Bahar	Special Volunteer	LCDB:NIDDK

**COOPERATING UNITS** (if any)

**LAB/BRANCH**

Laboratory of Cellular and Developmental Biology

**SECTION**

Biotechnology Unit, Office of the Chief

**INSTITUTE AND LOCATION**

NIDDK, NIH, Bethesda, Maryland 20892

**TOTAL STAFF YEARS:**

3

**PROFESSIONAL:**

2

**OTHER:**

1

**CHECK APPROPRIATE BOX(ES)**

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

**SUMMARY OF WORK** (Use standard unabbreviated type. Do not exceed the space provided.)

The Biotechnology Unit is responsible for:

- Large-scale production of procaryotes (bacteria), and eucaryotes (mammalian cells, insect cells).
- Large-scale recovery and purification of biologically active compounds (proteins, polysaccharides, etc.) from various sources.
- Process and method development associated with (1) bacterial growth, (2) eucaryotic cell growth, and (3) extraction and purification of biologically active compounds, especially proteins. The process development work is conducted to develop a procedure suitable for large-scale preparation and purification of material suitable for clinical trials.
- Research and development work not necessarily linked to a current process development project, but work that has long-term implications for fermentation processes and protein purification.

During the last 12 months, the Unit performed 24 different large-scale preparations, including micro-organisms (especially *E. coli*-carrying recombinant DNA) grown in volumes ranging from 5 to 300 liters, eucaryotic cells grown in volume up to 50 liters and processing of various biological materials.

After successful process development, the Unit put special effort into producing large quantities of two proteins needed for clinical trials in humans. The first is S antigen from bovine retina and the second is recombinant *Pseudomonas aeruginosa* exotoxin A, that was produced from recombinant *E. coli* BL21 DE3. In addition, the facility prepared large quantities of various human plasma cell lines for preparation and purification of peptides associated with HLA protein.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15503-12 LCDB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Regulation of Developmental Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Alan R. Kimmel	Research Chemist	LCDB:NIDDK
Others:	John L. Medabalimi	Senior Staff Fellow	LCDB:NIDDK
	Gail Ginsburg	Biologist	LCDB:NIDDK
	Rachel Gollop	Visiting Fellow	LCDB:NIDDK
	Jasmine Gruia-Gray	Visiting Fellow	LCDB:NIDDK
	Delwood Richardson	Senior Staff Fellow	LCDB:NIDDK

## COOPERATING UNITS (if any)

Dr. C. Londres, Membrane Regulation Section, LCDB:NIDDK

## LAB/BRANCH

Laboratory of Cellular &amp; Developmental Biology

## SECTION

Developmental Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL STAFF YEARS:

## PROFESSIONAL:

## OTHER:

5.5

5.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Two hormonally-regulated developmental systems are being investigated. Dictyostelium development is dependent upon signal transduction mediated by cell surface, cAMP receptor/G protein linkages. Here secreted cAMP acts extracellularly as a chemoattractant and primary signal to control cell motility, aggregation (multicellularity), cytodifferentiation, pattern formation and cell-type specific gene expression. We have previously isolated and disrupted 4 genes for distinct cAMP receptor subtypes, CARs 1, 2, 3 and 4. Analyses of CAR4-null cells indicate a role in cell differentiation and pattern formation. Differentiated cells localize to incorrect regions of the developing organisms. The promoters for CAR3 and CAR1 (early) expression have been dissected. We have identified short sequence regions within each that are required for their respective temporal, spatial and cAMP-regulated expression. The sequences exhibit specific binding to proteins isolated from nuclei at appropriate developmental stages. The zZIP1 protein of Dictyostelium possesses a leucine zipper and zinc fingers and is a presumed transcription factor. Dictyostelium carrying a disruption of the zZIP1 gene appear to have lost a subset of differentiated cells, the prestalk O cells, suggesting that zZIP1 is required for their differentiation or cell-type specific gene expression. The spiA gene appears to be a marker for terminal differentiation. We have used it to predict a gradient for spore differentiation and to examine the linkage between activation of cAMP dependent-protein kinase (PKA) and sporulation. We have also identified promoter sequences within spiA that are responsive to PKA activation.

The mammalian adipocyte is also regulated by hormone response. 3T3-L1 adipoblasts will differentiate into adipocytes when cultured with dexamethasone and insulin. During differentiation a variety of adipocyte markers become specifically expressed. One of these is perilipin. Previously we had shown that perilipin is an adipocyte protein located at the periphery of lipid droplets and had isolated cDNAs for perilipin. We now show that perilipin plays a significant role in the function of the adipocyte. Overexpression of perilipin appears to accelerate the rate of differentiation, whereas, antisense constructs used to block expression of perilipin appears to inhibit differentiation.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 DK 15505-16 LCDB

**PERIOD COVERED**

October 1, 1992 to September 30, 1993

**TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)**

Regulation of Adipocyte Metabolism

**PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)**

P.I.:	Constantine Londos	Research Chemist	LCDB:NIDDK
Others:	Andrew S. Greenberg	Senior Staff Fellow	LCDB:NIDDK
	Janice L. Theodorakis	IRTA Fellow	LCDB:NIDDK
	Christina Rondinone	Visiting Fellow	LCDB:NIDDK
	Daniel M. Levin	Biologist	LCDB:NIDDK
	Diane L. Servetnick	Biologist	LCDB:NIDDK
	Dawn L. Brasaemle	Senior Staff Fellow	LCDB:NIDDK

**COOPERATING UNITS (if any)**

A.R. Kimmel, J. Blanchette-Mackie, J. Gruia-Gray, LCDB:NIDDK; J. Wolff, LBP:NIDDK; R.P. Nordan, DCT:NCI; N. Edens, University of Maryland.

**LAB/BRANCH**

Laboratory of Cellular and Developmental Biology

**SECTION**

Membrane Regulation Section

**INSTITUTE AND LOCATION**

NIDDK, NIH, Bethesda, MD 20892

**TOTAL STAFF YEARS:**

9.5

**PROFESSIONAL:**

5.0

**OTHER:**

4.5

**CHECK APPROPRIATE BOX(ES)**

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

**SUMMARY OF WORK (Use standard structured type. Do not exceed the space provided.)**

This project focuses on the structure and function of adipocyte lipid storage droplets, especially the metabolism of stored triacylglycerols to release fatty acids, the primary energy reserve in animals. Intact lipid droplets were purified from differentiating 3T3-L1 adipocytes and methods were developed to purify the extremely solubilization-resistant droplet proteins, perilipin A and B, which appear to be the only major proteins located exclusively on the lipid droplet. Based on our earlier isolation of cDNAs for the rat perilipins, cDNAs for murine perilipins were isolated from a 3T3-L1 adipocyte library and full length coding sequences for perilipins A and B were obtained from a mouse genomic library; mouse and rat exhibit 93% predicted sequence identity. Further analysis of mRNAs suggests that expression of the A and B forms may be regulated independently.

Lipid hydrolysis in adipocytes involves translocation of hormone-sensitive lipase (HSL) to the lipid droplet surface following elevation of cAMP which lead to phosphorylation of HSL and the perilipins. We have succeeded in eliciting in vitro an A-kinase-dependent translocation of cytosolic HSL to purified lipid droplets. A role for perilipin in lipid hydrolysis is suggested by findings in cultured adrenal cells. Y-1 adrenal cells produce steroid upon elevation of cAMP by activating cholesteryl esterase, now thought to be identical to HSL of adipocytes. Western blotting and immunofluorescence reveals that perilipins A and B are associated with the lipid droplets in adrenal cells. Since perilipins are not expressed in numerous other tissues, the data strongly suggest a link between perilipin and lipid hydrolysis by HSL or HSL-like lipases. However, implicating perilipin in the maintenance of lipid droplet structure is the finding that perilipin ablation by transfection of adipoblasts with cDNA in the antisense orientation leads to an apparent dispersion of the lipid in numerous microdroplets, similar to the image produced by chronic polyphosphorylation of perilipin by A-kinase. Interestingly, over expression of perilipin by introduction of vectors bearing perilipin cDNA in the sense orientation leads to rapidly accelerated differentiation of adipocytes.

Two cytokines that have been implicated in disease-associated cachexia are TNF and interleukin-6 (IL-6). We have found that both act directly on cultured adipocytes and increase the tyrosyl phosphorylation of overlapping populations of proteins. Focusing on IL-6 actions, we have established that one of the major target proteins of this cytokine is MAP kinase, which is phosphorylated and activated within minutes by physiological concentration of the cytokine.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15506-09 LCDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Gene Expression in Mammalian Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Jurrien Dean	Section Chief	LCDB:NIDDK
Others:	Li-Fang Liang	Staff Fellow	LCDB:NIDDK
	Mary Familiari	Visiting Associate	LCDB:NIDDK
	Ann Ginsberg	Staff Fellow	LCDB:NIDDK
	Robert McIsaac	IRTA Fellow	LCDB:NIDDK
	Roxanne Chan	Visiting Fellow	LCDB:NIDDK
	Olga Epifano	Special Volunteer	LCDB:NIDDK
	Gail Osawa	Chemist, GS-11	LCDB, NIDDK

COOPERATING UNITS (if any)

Zhi-Bin Tong and Larry Nelson, DEB, NICHD; Malcolm Moos, DBB, CBER

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Mammalian Developmental Biology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

8.0

PROFESSIONAL:

7.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

The mouse ovary serves as a paradigm for investigating the developmental biology of mammalian gonadogenesis, oogenesis and fertilization.

Gonadogenesis: Mouse gestation occurs over 20 days. Germ cells, first detected in the developing embryo 7.5 days post coitus (dpc), migrate from the allantois to the genital ridge by 12.5 dpc. In XX females, the primitive gonad then differentiates into an ovary. RNA has been purified from female and male genital ridges isolated 12-13 dpc. A mRNA differential display technique is being used to identify female-specific gene products involved in early organogenesis of the ovary.

Oogenesis: The expression of the zona pellucida, an ovary-specific extracellular matrix composed of three glycoproteins (ZP1, ZP2, ZP3), serves as a marker of oocyte growth and differentiation in the adult female. The mouse and human ZP2 and ZP3 genes, transcripts and proteins have been characterized. A 12bp DNA sequence 200 bp upstream of the start of transcription of all four genes is necessary and sufficient for zona promoter activation of reporter genes microinjected into growing mouse oocytes. Efforts are currently underway to clone ZAP-1, the putative transcription factor that binds to this DNA element. These studies will provide important molecular details of mechanisms involved in the coordinate, oocyte-specific expression of the zona genes. ZAP-1 will additionally provide an early marker of oocyte growth and differentiation.

Fertilization: The three zona proteins are secreted and form an extracellular matrix that mediates the relatively species-specific events of fertilization. Using microinjection techniques to degrade endogenous zona transcripts and to introduce synthetic zona mRNAs, we are assessing protein-protein interactions in the assemblage of the zona matrix. To investigate the molecular biology of sperm-egg interactions mediated by the zona, we have established a mouse *in vitro* fertilization assay. We are using recombinant DNA techniques to express mouse and human zona proteins and to create mutant zona proteins, including mouse/human chimeric proteins. These will be tested for their ability to competitively inhibit fertilization, enabling establishment of structure-function correlations.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 DK 15508-05 LCDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Structure in Regulation of Mammalian Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Ann Dean	Research Chemist	LCDB:NIDDK
Others:	Qi-Hui Gong	Visiting Scientist	LCDB:NIDDK

COOPERATING UNITS (if any)

Randall Morse, LCDB:NIDDK; Genevieve Almouzni, LME:NICHD

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

4

PROFESSIONAL:

2

OTHER:

2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

The epsilon-globin gene is the first of the beta-like globin genes to be expressed during human development. It is actively transcribed until about the sixth week of development, in the large nucleated erythroid cells of the embryonic yolk sac. Expression of the beta-like-globin genes is under the control of the beta-globin locus control region (LCR), evolutionarily conserved sequences, encompassing erythroid specific DNase I hypersensitive sites (HS) in chromatin, 5-18 kilobases upstream of the epsilon-globin gene. Expression of the epsilon-globin gene in transgenic mice is dependent on beta-globin LCR enhancer sequences, and appropriate developmental regulation is observed when the epsilon-globin gene is linked to as little as HS I and HS II of the LCR.

Association of the beta-globin LCR with different globin promoters as they are sequentially expression during development is thought to occur through stable complexes formed by chromatin binding proteins. Using *in vitro* protein-DNA binding studies, clustered point mutations, and transient expression assays with a reporter gene, have identified a number of transcription factors that can be expected to participate, *in vivo*, in mediating the interaction of the LCR with the epsilon-globin gene. These proteins include the ubiquitous transcription factor Sp1, and the erythroid transcription factors NF-E2 and GATA-1. To study these interactions in the context of chromatin we have assembled DNA templates containing the regulatory elements with histones and examined the effect on *in vitro* transcription. Naked DNA templates support abundant transcription, even in the absence of the enhancer. Assembly of the templates repressed transcription, but there was a diminution of this effect when the enhancer was present in the template. Using a different approach, we have constructed a minichromosomal vector to probe the interaction of the epsilon-globin gene promoter and enhancer in chromatin *in vivo*. The minichromosomes contain a marked epsilon-globin gene. They are stably maintained in several human tissue culture cell lines where they are assembled into chromatin. Our analysis of RNA produced by individual clones of embryonic/erythroid K562 cells, shows that expression of the episome-borne epsilon-globin gene is dependent on the presence of the beta-globin LCR *in cis*: the simple availability of erythroid transcription factors is insufficient to allow expression. The minichromosome system may provide a means to study the effects of the LCR on chromatin structure, as well as its enhancer activity.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

701 DK 15509-02 LCDB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of Polyprotein Processing in Retroviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	John L. Medabalimi	Senior Staff Fellow	LCDB:NIDDK
	Ewald M. Wondrak	Visiting Associate	LCDB:NIDDK
	Alan R. Kimmel	Research Chemist	LCDB:NIDDK

COOPERATING UNITS (if any)

Drs. N.T. Nashed and D.M. Jerina, Section on Oxidative Mechanisms, LCB/NIDDK; Drs. K. Parris and D. Davies, LMB/NIDDK; Drs. K. Sakaguchi and E. Appella, LCB/NCI

LAB/BRANCH

Laboratory of Cellular and Developmental Biology

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

The purified 52 kDa polyprotein (MBP-12AA-Protease-19AA) containing 12 amino acids of the transframe protein and 19 amino acids of the reverse transcriptase flanking the N and C terminus of the HIV-1 protease, respectively, upon 10 fold dilution, undergoes time dependent autoprocessing to release the 11 kDa mature protease in two steps. The initial step involves the cleavage at the N terminus of the protease to produce the protein species MBP-12AA (39 kDa) and Protease-19AA (13.2 kDa), followed by a slower cleavage of the 13.2 kDa protease intermediate leading to the release of the 11 kDa mature protease. The inhibition of the autoprocessing reaction with a peptide substrate of the mature protease and pepstatin A, a specific inhibitor of aspartic acid proteases indicates that the active site of the dimeric protease is fully formed in the renatured polyprotein. The autoprocessing reaction also requires a catalytic group with an apparent pK(a) of 6.7 in its protonated form, as does the hydrolysis of peptide substrates by the mature protease. The fragmentation of the polyprotein is concomitant with an increase in protease activity. Initial rates of the first-step of the reaction are linearly dependent on the protein concentration indicating that the reaction is first-order in protein concentration monitored by following the disappearance of the polyprotein and appearances of the MBP-12AA and enzymatic activity. The first-order rate constant for the slowest step on the protein folding pathway is 15 times faster than the first cleavage on the autoprocessing pathway. Thus the folding and dimerization of the polyprotein is not a rate limiting step in the autoprocessing reaction. The rate of appearance of enzymatic activity is identical to that of the appearance of MBP-12AA and to the disappearance of the MBP-12AA-Protease-19AA and to the sum of the accumulation of the 13.2 kDa Protease-19AA and 11 kDa protease. This indicates that the 13.2 kDa protein has enzymatic activity comparable to that of the mature 11 kDa protease. These results are discussed in the framework of a mechanism in which homodimerization of the protease domains of the fusion protein must form prior to the rate-limiting intramolecular cleavage of the N-terminal strands leading to the appearance of mature like protease activity. We have also characterized novel protease cleavage sites within the 72 amino acid nucleocapsid precursor (p7/p1) and the first zinc binding domain of the mature 55 amino acid nucleocapsid protein (p7). The kinetic parameters of these cleavages were determined. The rate of hydrolysis of the p7/p1 scissile bond was not affected by the conformation of the p7 domain induced as a result of zinc binding, whereas, the cleavage within the p7 protein was inhibited when complexed with zinc.



ANNUAL REPORT  
OF  
THE LABORATORY OF BIOCHEMISTRY AND METABOLISM  
NATIONAL INSTITUTE OF DIABETES AND  
DIGESTIVE AND KIDNEY DISEASES

The Laboratory conducts research in several areas of the biochemical sciences that include morphogenesis, development, endocytosis, endocrinology, membrane transport, detoxication, and the physical and chemical behavior of proteins and nucleic acids. It does so by applying a broad array of different approaches. Resolution is being attempted by methods that stem from biochemistry, carbohydrate chemistry, cell biology, genetics and molecular biology. Although seemingly diverse, the common element to each of the research areas is appropriate to the Laboratories' designation: biochemical, metabolic and physical approaches are being brought to bear on major problems encompassed by the Institute's charge. It is the close proximity of experienced investigators from diverse scientific disciplines, able to discuss their individual problems with each other, that provides synergistic effects for resolution of the questions under investigation.

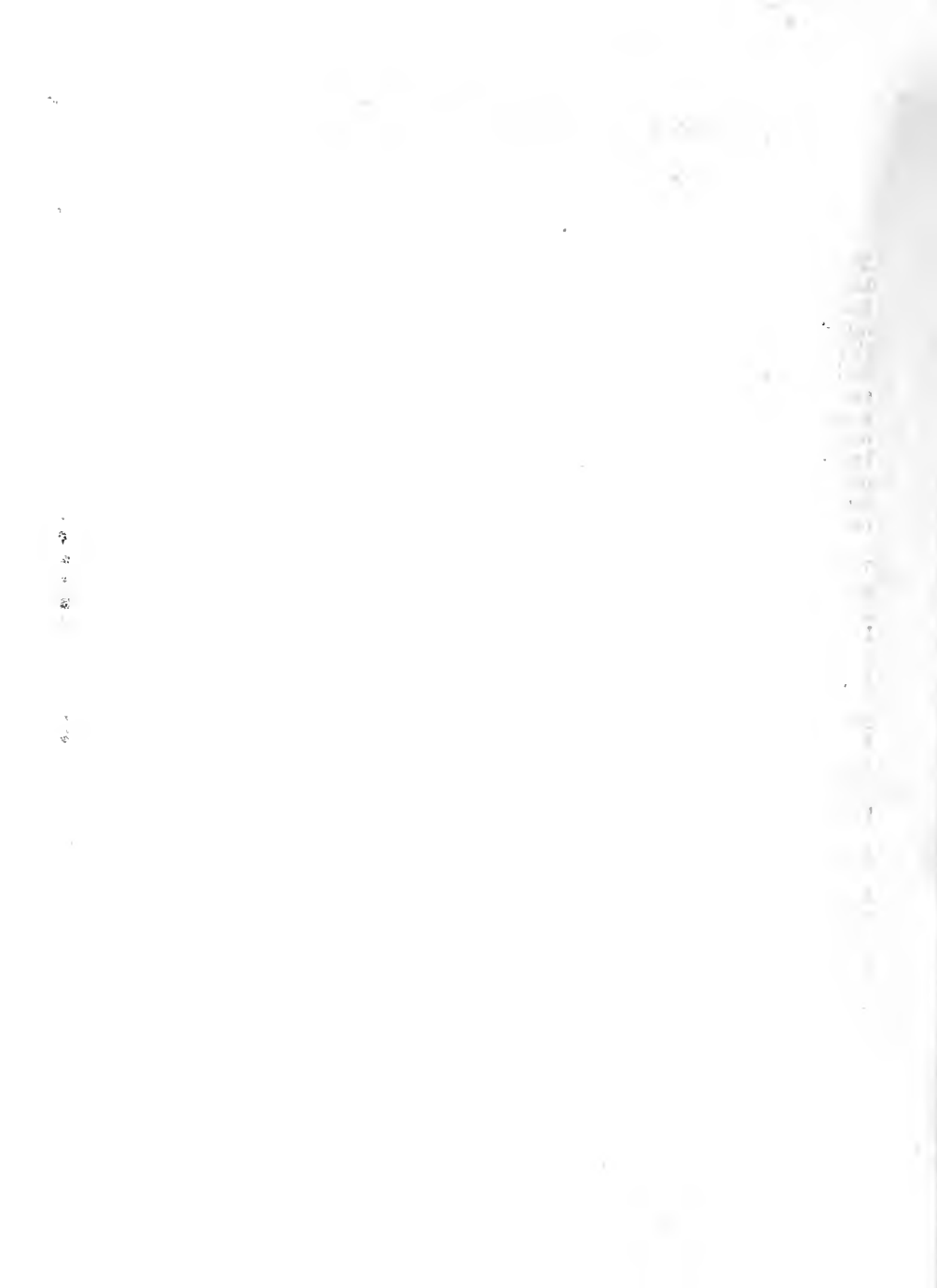
The year saw the transfer of Dr. Peter McPhie to LBP of this Institute although he continues to collaborate with several members of LBM. Similarly, Dr. Donald Rau and Dr. Adrian Parsegian, who formed the Biophysics unit within the Laboratory, will transfer at the end of the fiscal year.

#### I. ENZYMES: FUNCTIONAL AND ABNORMAL

Several groups are active in this broadly designated area which covers the search for genes of lysosomal enzymes whose absence leads to disease, and a genetic approach to the basic catalytic mechanism that is responsible for the sulfation of otherwise toxic substances and the means by which enzymes are folded into the proper, active configuration.

##### Inherited Disorders of Lysosomal Function

N-acetyl-neuraminidase is a lysosomal enzyme deficient in the inherited lysosomal storage disorders, sialidosis and galactosialidosis. Both diseases are clinically heterogeneous exhibiting both mild and severe forms. The former disease is thought to arise from mutations in the structural gene coding for neuraminidase. The latter disease results from a defect in a glycoprotein called protective protein which appears essential for maintenance of activity of both neuraminidase and  $\beta$ -galactosidase. All three proteins copurify and are believed to exist as a functional complex within the lysosome. Three approaches are being followed to clone the mammalian neuraminidase. (1) The complex has been purified from bovine testicular tissue for use in isolation of neuraminidase to obtain amino acid sequence information for cloning purposes. (2) A set of degenerate primers based on a five amino acid sequence motif found in viral,





bacterial and trypanosome neuraminidases has been used to PCR amplify mRNA from human fibroblasts in attempts to capture an authentic cDNA fragment coding for neuraminidase. (3) An 80,000 bp segment of DNA located on chromosome 6 in the class III gene region of the major histocompatibility complex believed to contain the gene for neuraminidase, has been isolated and attempts are in progress to trap the neuraminidase exons for use in screening a cDNA library. This third approach is based on the following; a) a study that describes a single patient exhibiting the clinical features of both sialidosis and congenital adrenal hyperplasia along with the corresponding enzyme deficiencies of neuraminidase and 21-hydroxylase, thereby suggesting a location for the neuraminidase gene proximal to that of the 21-hydroxylase gene on chromosome 6 in the class III gene region of the major histocompatibility complex, and b) a suggestion of the presence of the sequence motif found in viral, bacterial and trypanosome neuraminidase in a newly discovered cluster of genes neighboring the 21-hydroxylase gene.

### Enzymatic Basis of Detoxication

As part of the project in which the enzymes of detoxication are being examined as to their catalytic mechanism, a specific sulfotransferase that is active with phenols has been closely investigated. The protein, tyrosine-ester sulfotransferase, is listed by the Nomenclature Committee of the IUBMB as an enzyme for the transfer of the sulfonyl group of 3-phosphoadenosine-5-phosphosulfate to a wide variety of phenols. Indeed, one of the characteristics of the enzymes that are active with foreign compounds, the enzymes of detoxication, is the broad range of compounds that are accepted as substrates. This laboratory has cloned the enzyme from rat liver mRNA and has expressed it in *Escherichia coli* in very large quantities. The resultant tyrosine-ester sulfotransferase expressed by the bacterium differs in several properties from that in the natural enzyme. In fact, there are differences in substrate specificity, in pH optima for specific substrates, and even in the resistance of the enzyme to heat. Despite the number of trivial possibilities that exist for this difference, most of them have been eliminated and the current view is that the recombinant enzyme is folded slightly differently from the natural one. Although such characteristics as substrate specificity, pH optimum and heat sensitivity have been used to characterize differences among specific enzymes, these results point clearly to the need for genetic data before concluding that isoforms with overlapping specificity exist as specific enzyme species on the basis of such properties.

## II. MORPHOGENESIS AND DEVELOPMENT

At a higher level of organization, one can question how enzymes or regulatory genes affect cell structures or the function of entire glands.

### Polysaccharides in Morphogenesis

Two major carbohydrate components of the yeast cell are under investigation. One is chitin. Experiments with synchronized cultures have confirmed that the

2

7  
5

7

2

26

yeast chitin synthetases are regulated at the post-translational level, although regulation by synthesis and inactivation may also play a role with chitin synthetase 2. Progressive deletions in the *CHS2* gene have defined a sequence that is critical for activity and function of the corresponding chitin synthetase. A hybrid gene containing that sequence linked to a portion of the *CHS1* gene, led to expression of a Chs1-like synthetase that has lost the zymogen characteristic. The second major structural component of the yeast cell wall is  $\beta(1\rightarrow3)$ glucan. Purification of a component of  $\beta(1\rightarrow3)$ glucan synthetase, followed by photolabeling, led to the identification of a 20 kDa protein as the GTP-binding subunit of this fraction.

Previous work has suggested that the components of the yeast cell wall are covalently linked to each other. In particular, chitin is attached to  $\beta(1\rightarrow3)$ glucan. Digestion of yeast cell walls with  $\beta$ -glucanase and chitinase followed by reduction with borotritide and chromatography on sizing columns led to the isolation of oligosaccharides containing both N-acetylglucosamine and glucose residues. These oligosaccharides contain the previously postulated linkage between chitin and glucan. Because the oligosaccharides are absent in a chitin synthetase 3-deficient strain, it is concluded that chitin synthetase 3 catalyzes the synthesis of the chitin that is attached to glucan.

#### Tissue Specific and Hormone Regulated Gene Expression

Development and differentiation of the mammary gland during puberty, pregnancy and lactation is controlled by hormones and growth modulators. This laboratory had shown that an endogenous milk protein, the whey acidic protein (WAP), can participate in mammary development. Temporal deregulated expression in mammary tissue of transgenic mice resulted in abrogated mammary development. Ectopic expression of the WAP gene under the control of the MMTV-LTR resulted in impaired mammary development as well as neoplasia in the coagulation gland. No growth aberrations were observed in such other expressing tissues as the salivary gland. This suggests that WAP exerts its growth modulatory effects in a cell-specific manner.

The role of a given protein in tissue formation and differentiation processes can be evaluated using gain of function experiments in transgenic animals. Current systems, however, do not permit a spatial and temporal controlled activation and inactivation of transgenes. Such a system has now been established using viral and bacterial tools. A binary system was built in which gene activity can be modulated using tetracycline. One line of mice carries a transgene composed of the human cytomegalovirus (HCMV) enhancer, the coding region for the DNA binding domain of the tetracycline repressor, and the transcriptional activation domain VP16 from the Herpes simplex virus. The other line of transgenic mice carries a reporter gene composed of tetracycline operators next to a minimal promoter and the luciferase gene. Upon breeding, double transgenic mice have an activated reporter gene. Activity of this reporter gene should be subject to modulation by tetracycline, an investigation that is currently in progress.



### III. PROTEIN SORTING AND TRANSPORT FUNCTIONS

Central to modern biology is the nature of the mechanism for the movement of macromolecules, glycoproteins in particular, not only into and out of the cell but also into specific organelles. The mechanisms involved are being sought from the approaches of the disciplines of somatic cell genetics, molecular biology, carbohydrate chemistry, endocrinology and biochemistry. The implications of the work extend from cell biology to applications in thyroid pathobiology and approaches to the AIDS virus.

#### Role of Carbohydrate Moiety of Glycoproteins

Studies of the role of carbohydrates in biological systems have been extended. Previously described findings on the hormonal regulation of sialylation in rat thyroid cells have been examined in human thyroglobulin isolated from patients with Graves disease and from patients exhibiting endemic goiter. In contrast to normal controls or material from endemic goiters, the thyroglobulin recovered from patients with Graves disease was characterized by severe hypersialylation and aberrant localization of the sialic acid residues within the carbohydrate core. As a separate investigation, techniques designed to isolate and characterize individual nuclear protein fractions have revealed the presence of enzymatically degradable glycogen in the pore-lamina fraction of the nucleus and the presence of one or more complex glycoproteins within the nuclear matrix.

#### Role of the Nuclear Envelope in Intracellular Protein Sorting

Transport across the nuclear pore complex is essential for regulating cell growth and normal development. The structure of the nuclear pore and its involvement in nuclear transport are being studied at a molecular level. The nuclear pore complex is made up of a family of phosphorylated glycoproteins having covalently attached O-linked N-acetylglucosamine. The major nuclear pore glycoprotein p62 was expressed by this group in bacteria, and its glycosylation *in vitro* was examined. Glycosylated p62 is also being obtained by expression in insect cells using baculovirus. The sites of glycosylation of p62 have been determined by deletion analysis and protease mapping. The enzyme responsible for glycosylation of the nuclear pore glycoproteins has been partially purified from rabbit reticulocyte lysate; an attempt is being made to clone the cDNA of this enzyme. To examine the function of the nuclear pore glycoproteins in *in vitro* transport, *Xenopus laevis* extracts capable of nuclear assembly and transport have been employed. Using this system, it was demonstrated that the O-linked N-acetylglucosamine moiety of nuclear pore glycoproteins can be modified without altering nuclear transport. These extracts were also used to demonstrate that the glycosylation and phosphorylation of nuclear pore glycoproteins change during the cell cycle. The findings suggest that there may be a coordinated regulation of glycosylation and phosphorylation which accompany the breakdown and reassembly of the nuclear pore during the cell cycle.



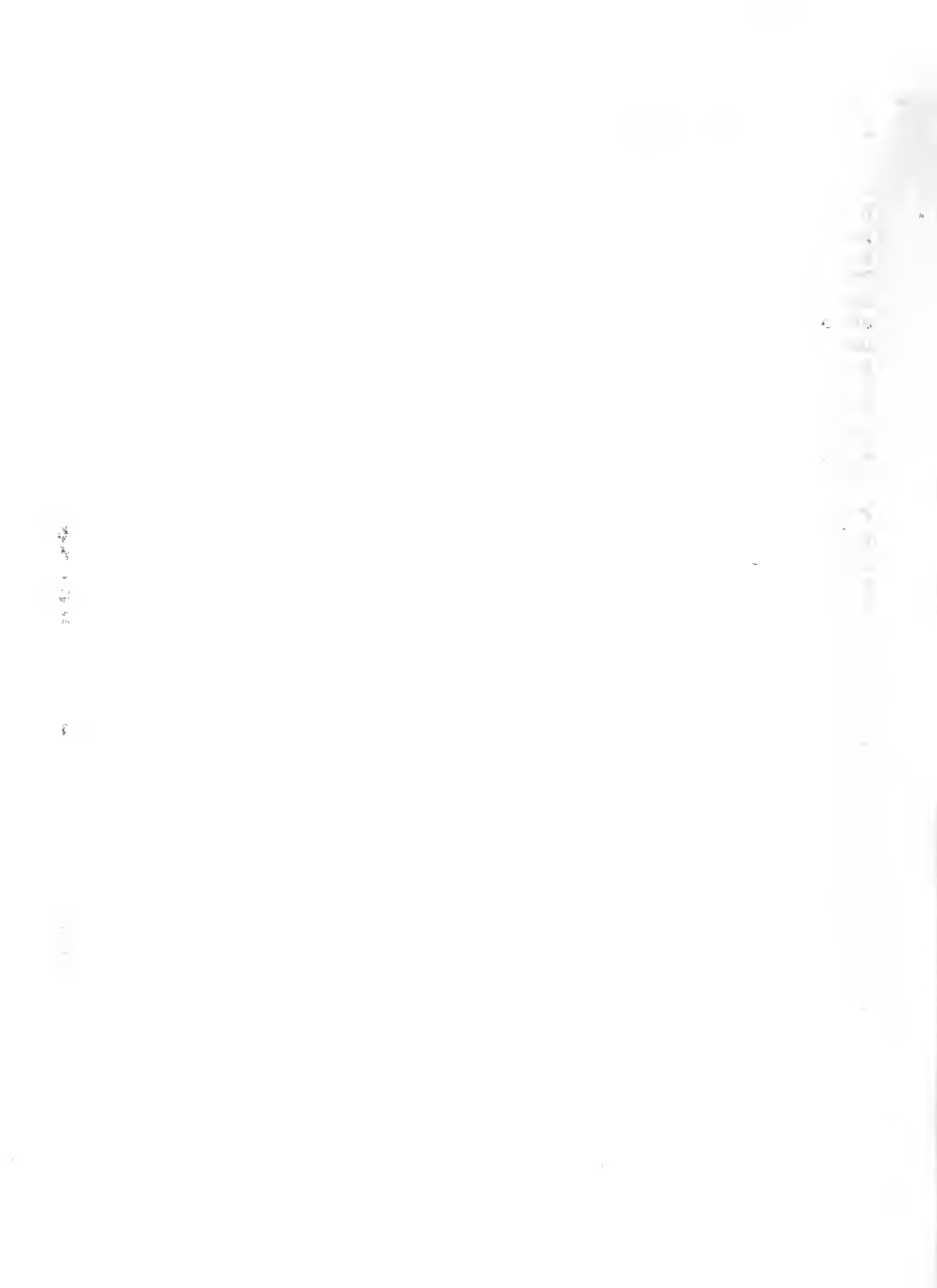
### Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

The work continues to emphasize iodide transport in thyroid and the relationship of thyroglobulin sialylation and iodination necessary for normal thyroid hormone formation. The project has developed further in the cloning of iodide transport proteins from thyroid. For the project, a cDNA expression library, prepared from cultured rat thyroid cells superinduced to actively transport iodide, is being used. The screening method uses a replica plate assay to detect iodide transport in a mutant line of rat thyroid cells that responds to thyrotropin, as demonstrated by elevations in cyclic AMP levels, but does not transport iodide. The particular methods for cloning were selected because of the lack of success using more traditional methods for cloning. Previous selection of iodide transport clones in this laboratory were based on stilbene binding, a property associated with iodide loss from the thyroid into the follicular lumen. These clones are related to non-erythroid band 3 proteins. If the reconstitution of both types of iodide carriers (a sodium/iodide symporter and an iodide efflux channel) is successful, studies of the properties of iodide transport in thyroid will be more productive.

Collaborative studies demonstrated the role of thyrotropin in sialylation of thyroglobulin in cultured rat thyroid cells. These studies are now extended to address the glycosylation of thyroglobulin prepared from thyroid tissue from patients with two different thyroid diseases. In Graves' disease where thyroid hormone levels are elevated, thyroglobulin is found to be both hyper- and abnormally sialylated. This contrasts with thyroglobulin isolated from endemic goiter which is both hypo-iodinated and hypo-glycosylated. These findings continue to support a significant role for sialylation in thyroglobulin secretion and iodination leading to normal thyroid hormone formation.

### Cell Regulation by Hormones, Growth Factors, Autoantibodies, and Oncogenes

One group is studying the structure-function relationships of the thyrotropin (TSH) receptor, the involvement of this receptor in autoimmune thyroid disease, as well as the relationship between thyroid autoimmune diseases and other organ-specific autoimmune diseases, i.e. lupus and diabetes. Structure-function relationships of the TSH receptor are being compared with other glycoprotein hormone receptors. The group is evaluating the interdependent regulation of thyroid function and growth by the TSH receptor and receptors for other ligands: gonadotropins, adrenergic, cholinergic, insulin, insulin-like growth factors (I and II), fibroblast growth factors, hydrocortisone, thyroid hormones, purinergic, bacterial toxins (cholera, pertussis, tetanus), interferon, and cytokines. Particular attention is given to identifying determinants on the receptors important for TSH and receptor autoantibody binding and signal transduction, as well as the transcriptional and posttranscriptional mechanisms by which TSH and the other receptors affect gene expression in thyroid cells. The relationship between oncogene transformation (thyroid tumors and adenomas), the development of autoimmunity, and the loss of normal regulation of thyroid function and growth is being investigated. The role of different signal transduction mechanisms - cAMP, Ca/phosphoinositide and arachidonate - in





thyroid cell growth and differentiated function is being studied. The role of membrane lipids in regulation of TSH receptor expression, LDL receptor expression, and cholesterol biosynthesis are being evaluated, and the role of major histocompatibility antigens in the development of autoimmune thyroid diseases and organ specific immune diseases are under study. Under development are human thyroid and islet cells which can grow in continuous culture, act as models of endocrine and thyroid disorders, and serve as donor cells for transplantation or gene therapy situations. These studies combine a molecular biology, cell biology and monoclonal antibody approach.

### Endocytosis, Secretion and Compartmentalization

Association of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase with other macromolecules and cellular structures (e.g. Band 3, microtubules, triad junctions) has been frequently reported. The functional significance, if any, of these associations is unknown. A single amino acid change in this enzyme from a mutant Chinese hamster ovary cell (FD1.3.25) results in an association of that protein with microtubules that cannot be disrupted by agents (e.g. high salt, ATP) that effect dissociation of the normal dehydrogenase; this correlates with genetically dominant alterations in endocytosis and aberrant association of late endosomes with microtubules observed in FD1.3.25. FD1.3.25 is a heterozygote, with normal and mutated protein alleles; based on steady-state RNA levels, the mutated form represents 25% of the total enzyme. Moreover, the enzyme is a tetramer. Thus, very little of the dehydrogenase from FD1.3.25 is of "purely mutant" form. In order to study the properties of the mutant enzyme, dehydrogenase cDNA bearing the FD1.3.25 mutation has been overexpressed in E. coli.

### Intracellular Traffic in HIV Infection

Transport across the nuclear membrane is necessary during the life cycle of HIV. Once in the cytoplasm, HIV RNA is converted to double stranded DNA which must enter the nucleus. Viral regulatory proteins enter the nucleus; viral transcripts are exported into the cytoplasm. The viral REV and TAT proteins have been identified as key regulators of the transcription and transport of HIV envelope mRNA. These proteins contain sequences which target them to the nucleolus. Recently, it has been demonstrated that a reduction in cellular GTP levels induced by inhibitors of IMP dehydrogenase, reduce the nucleolar accumulation of other nucleolar proteins. These inhibitors, which include ribavirin or mycophenolic acid, are being tested for their ability to interfere with HIV TAT and REV nucleolar localization. A means has also been devised for generating nuclei in vitro around exogenously added DNA. The method uses extracts from Xenopus laevis eggs. The nuclei assembled in such extracts mimic interphase nuclei in many ways and carry out active nuclear transport. These reformed nuclei have structures resembling nucleoli (termed pre-nucleoli). These preparations should allow the mechanism of REV action and the movement of other molecules involved in the HIV life cycle to be examined *in vitro*. In other studies the structure of the nuclear pore has been examined. The nuclear pore requires glycoprotein components for proper morphology and function.



#### IV. HYDRATION FORCES

The more physical interactions of macromolecules -- particularly proteins, lipids and DNA -- with their environment is being studied with special focus on the role of water.

##### Direct Measurement of Forces between Membranes or Macromolecules

The ability to measure directly the forces between membranes or between macromolecules is creating a new logic for thinking about molecular recognition, and folding. The outstanding feature of interaction is that as molecules or membranes approach contact, the important work of approach involves removal of organized water solvent from the apposing surfaces. These "hydration forces" are now recognized to act in materials as diverse as lipid bilayers, proteins, DNA double helices, and stiff polysaccharides. Recently the intermolecular forces in native and reconstituted collagen fibers have been measured at various temperatures, pH, ionic conditions, and in the presence of several small solutes. It has been shown that salt does not fully penetrate into the space between collagen triple helices. Osmotic pressure applied from outside by the excluded salt is an important component of collagen fiber assembly. Force measurements have demonstrated that temperature-favored assembly of the fibers is driven by water-mediated hydrogen bonding between the apposing polar residues rather than by the hydrophobic effect, usually invoked to explain assembly of proteins. One can now think of a competition between repulsive and attractive hydration forces, depending on how well protein surfaces match each other.

A dependence of hydration forces between DNA molecules on small solutes present in the solution has been studied. An unusual re-entrant liquid-gel-liquid phase transition sequence, observed during measurement of forces between didodecylphosphate bilayers, has been explained. The osmotic stress technique for measuring forces between spherical particles has been developed.

##### Physics of Ionic Channels and other Proteins with Aqueous Cavities

The kinetics of Staphylococcus aureus alpha-toxin channels were examined by "noise" analysis at several different pH's to measure the kinetics of proton binding to ionizable sites within the channel. Recognizing that this binding follows first-order kinetics, and noting that proton binding creates a step change in channel conductance, one sees that the responsible ionizable sites have a pK of 5.5 and that the association and dissociation rate constants are  $8 \times 10^9$  and  $10^5$  respectively. These values imply that the ionizable residues are freely accessible to the aqueous phase and that the active groups are either histidine, glutamic acid or aspartic acid.

By exposing ionic channels to neutral polyethyleneglycols of different molecular weight, it has been possible to generalize the use of water-soluble polymers to probe channel structure. Large, excluded solutes in membrane-bathing solutions create an osmotic work of transition between different conductance states, a work proportional to the difference in polymer-inaccessible aqueous spaces of these



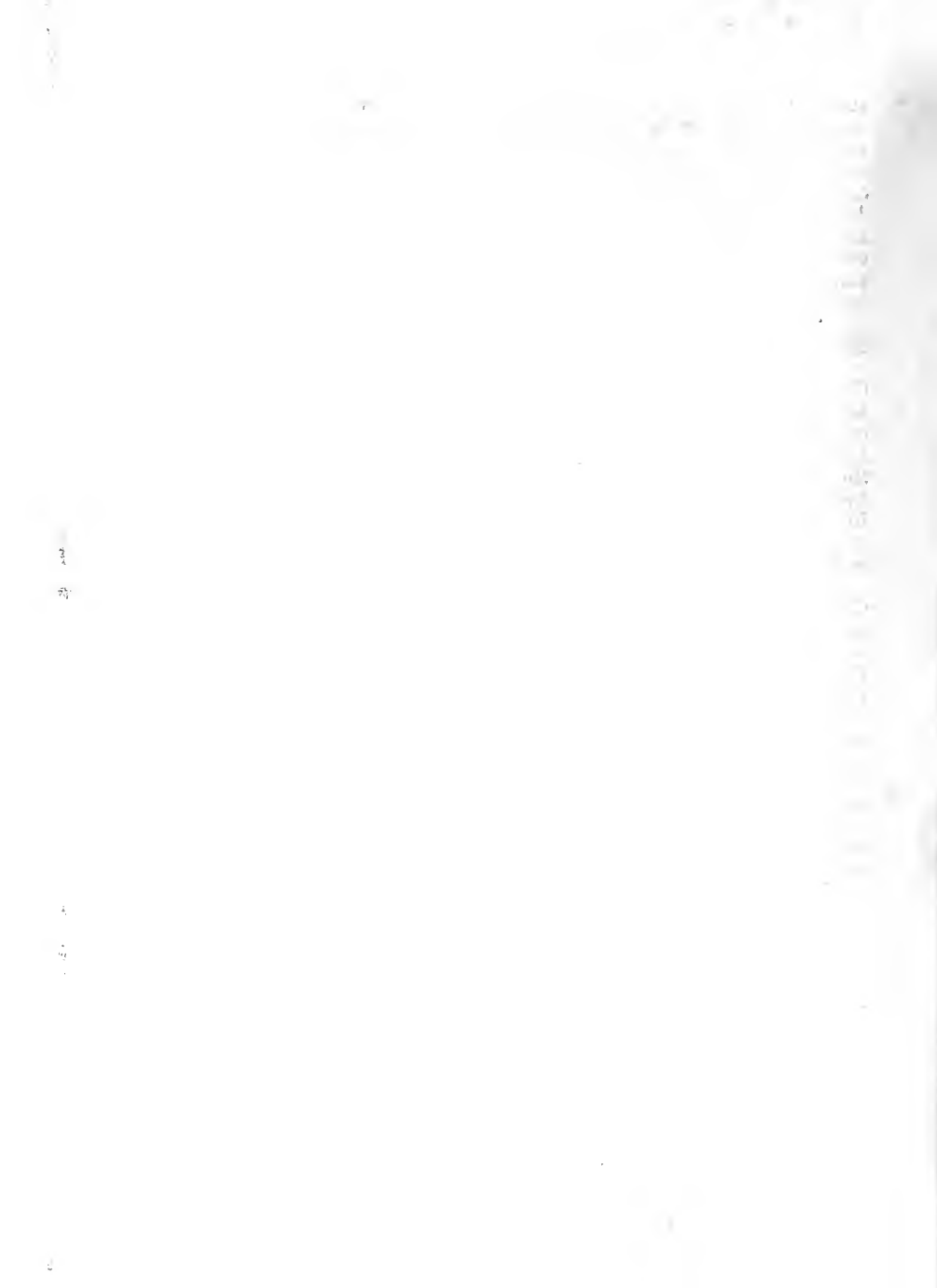
states. Progressively smaller polymers begin to penetrate the channel space, to cause a decrease in channel conductance and to exert a correspondingly reduced effective osmotic pressure. We find that the channel, a kind of ultimate molecular sieve, appears to exclude polymers on the basis of their rotational volume calculated from the radius of gyration (rather than the polymer cross-section or diameter typically used to gauge channel size). In the particular case of alamethicin, probing of the polymer reveals a fixed (about  $3,000 \text{ \AA}^3$ ) aqueous volume change with each successive step between the five different conductance states. These states, then, are collections of conducting units of nearly the same conductance rather than pores of successively larger radius.

### Structure and Physical Properties of DNA and DNA-Protein Complexes

Changes in water binding upon complex formation have been measured for several specific sequence DNA binding proteins. Large numbers of water molecules are displaced in the binding reaction: ~ 100 for the binding of the galactose operon repressor, ~ 50 for cAMP receptor protein (CAP), and ~ 30-40 for the specific binding of the restriction nuclease Eco RI. These changes in hydration correlate approximately with the size of the DNA recognition sequence. In addition, the binding of a second galactose operon repressor to the naturally occurring 2 operator DNA fragment shows an additional release of water due to protein-protein interactions.

Changes in hydration accompanying the binding of analogs of the antibiotic netropsin to specific DNA sequences are being measured. A correlation between water release and binding strength to different sequences has now been observed. The complete set of data of water release and binding energy for many different sequences will allow a quantitative analysis of the importance of the hydration forces observed in condensed systems for the specific binding of peptides and proteins to DNA in dilute solution.

The electric birefringence of *Acanthamoeba* myosin II filaments has revealed a large change in LMM-HMM junction flexibility with ATP binding to myosin heads. In addition to the well established crossbridge motions of myosin heads, a concomitant change in filament stiffness coupled to ATP binding and hydrolysis appears involved in the force generation cycle of myosin.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17001-27 LBM

## PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of Carbohydrate Moiety of Glycoproteins in Cellular Activity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G. Ashwell Institute Scholar LBM, NIDDK

Others: W. Berlin IRTA LBM,  
NIDDK O. Gabriel Parttime Special Volunteer LBM, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

2.0

## PROFESSIONAL

2.0

## OTHER

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The work carried out this year represents a continuation and extension of earlier studies on the role of carbohydrates in biological systems. Two separate areas are covered in this report. (a) The previously described findings on the hormonal regulation of sialylation in rat thyroid cells have been examined in human thyroglobulin isolated from patients with Graves disease and from patients exhibiting endemic goiter. In contrast to normal controls or material from endemic goiters, the thyroglobulin recovered from patients with Graves disease was characterized by severe hypersialylation and aberrant localization of the sialic acid residues within the carbohydrate core. (b) Techniques designed to isolate and characterize individual nuclear protein fractions have revealed the presence of enzymatically degradable glycogen in the pore-lamina fraction of the nucleus and the presence of one or more complex glycoproteins within the nuclear matrix.

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1900-1901



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17002-23 LBM

## PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enzymatic Basis of Detoxication

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.B. Jakoby Chief, LBM LBM, NIDDK

Others: Y-S. Yang Visiting Fellow LBM, NIDDK  
X. Chen Visiting Fellow LBM, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Enzymes and Cellular Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

4.5

4

0.5

## CHECK APPROPRIATE BOX(ES)

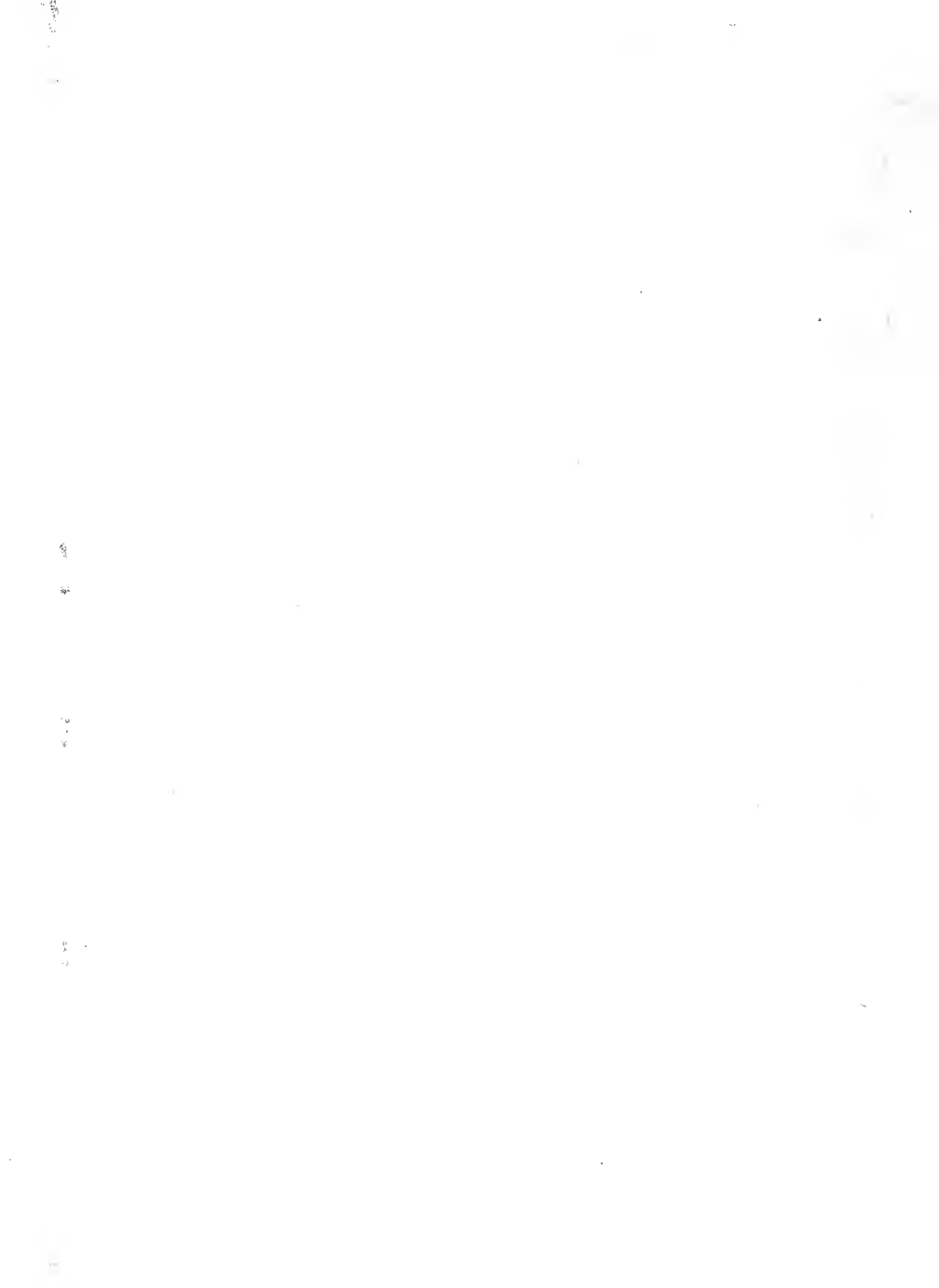
- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

As part of the project in which the enzymes of detoxication are being examined as to their catalytic mechanism, a specific sulfotransferase that is active with phenols has been closely examined. The enzyme, tyrosine-ester sulfotransferase, is listed by the Nomenclature Committee of the IUBMB as an enzyme for the transfer of the sulfonyl group of 3-phosphoadenosine-5-phosphosulfate to a wide variety of phenols. Indeed, one of the characteristics of the enzymes that are active with foreign compounds, the enzymes of detoxication, is the broad range of compounds that one accepted as substrates.

This laboratory has cloned the enzyme from rat liver mRNA and has expressed it in *Escherichia coli* in very large quantities. The resultant tyrosine-ester sulfotransferase expressed by the bacterium differs in several cogent properties from that in the natural enzyme. In fact, there are differences in substrate specificity, in pH optima for specific substrates, and even in the resistance of the enzyme to heat. Despite the number of trivial possibilities that exist for this difference, most of them have been eliminated and the current view is that the recombinant enzyme is folded slightly differently from the natural one.

Although such characteristics as substrate specificity, pH optimum and heat sensitivity have been used to characterize the differences among specific enzymes, it should be clear that genetic data is required before concluding that isoforms with overlapping specificity exist as specific enzyme species on the basis of these properties.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17003-26 LBM

## PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Polysaccharides in Morphogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E. Cabib Senior Research Chemist LBM, NIDDK

Others: P.C. Mol Visiting Fellow LBM, NIDDK  
 J.A. Shaw Staff Fellow LBM, NIDDK  
 W.-J. Choi Visiting Fellow LBM, NIDDK  
 Roman Kollar Special Volunteer LBM, NIDDK  
 Emanuela Lacanà Visiting Fellow LBM, NIDDK

## COOPERATING UNITS (if any)

LCB, NHLBI (Blair Bowers); Departamento de Microbiologia, University of Salamanca, Spain (Angel Duran); Slovak Academy of Sciences, Institute of Chemistry, Bratislava, Czechoslovakia (Vladimir Farkas); CRADA, American Cyanamid, Princeton, NJ

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Morphogenesis

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.5

## PROFESSIONAL:

5.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Experiments with synchronized cultures have confirmed previous indications that the yeast chitin synthetases are regulated at the post-translational level, although regulation by synthesis and inactivation may also play a role in the case of chitin synthetase 2. Progressive deletions in the *CHS2* gene have defined a sequence that is critical for activity and function of the corresponding chitin synthetase. A hybrid gene containing that sequence linked to a portion of the *CHS1* gene led to expression of a Chs1-like synthetase that has lost the zymogenic character.

$\beta(1\rightarrow3)$ glucan is the major structural component of the yeast cell wall. Purification of a component of  $\beta(1\rightarrow3)$ glucan synthetase followed by photolabeling led to the identification of a 20 kDa protein as the GTP-binding subunit of this fraction.

Previous work has suggested that the components of the yeast cell wall are covalently linked to each other, in particular that chitin is attached to  $\beta(1\rightarrow3)$ glucan. Digestion of yeast cell walls with  $\beta$ -glucanase and chitinase followed by reduction with borotritide and chromatography on size columns led to the isolation of oligosaccharides containing both N-acetylglucosamine and glucose residues. These oligosaccharides contain the previously postulated linkage between chitin and glucan. Because the oligosaccharides are absent in a chitin synthetase 3-deficient strain, it is concluded that chitin synthetase 3 catalyzes the synthesis of the chitin that is attached to glucan.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17004-25 LBM

## PERIOD COVERED

October 1, 1992 through September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thermodynamic and Kinetic Studies of Protein Structure and Enzymatic Mechanisms

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. McPhie

Research Chemist

LBM, NIDDK

## Others:

## COOPERATING UNITS (if any)

LMC, NHLI (Robert Adelstein); LCP, NIDDK (Edith Miles); MB, NCI (Jane Cheng); LPS, DCRT (Richard Shrager)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS: 0

PROFESSIONAL: 0

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project was transferred to LCP, NIDDK April 5, 1993.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17008-10 LBM

## PERIOD COVERED

October 1, 1992 through September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of the Nuclear Envelope in Intracellular Protein Sorting

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J.A. Hanover Research Chemist LBM, NIDDK

Others: S. Bailer Visiting Fellow LBM, NIDDK  
 W. Lubas Research Associate LBM, NIDDK  
 M. Miller IRTA LBM, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Cell Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

3.5

## PROFESSIONAL

3.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Transport across the nuclear pore complex is essential for regulating cell growth and normal development. The structure of the nuclear pore and its involvement in nuclear transport are being studied at a molecular level. The nuclear pore complex is made up of a family of phosphorylated glycoproteins having covalently attached O-linked N-acetylglucosamine. We have expressed the major nuclear pore glycoprotein p62 in bacteria and examined the glycosylation in vitro. Glycosylated p62 is also obtained by expression in insect cells using baculovirus. The sites of glycosylation of p62 have been determined by deletion analysis and protease mapping. The enzyme responsible for glycosylation of the nuclear pore glycoproteins has been partially purified from rabbit reticulocyte lysate; an attempt is being made to molecularly clone this enzyme.

To examine the function of the nuclear pore glycoproteins in vitro, transport, Xenopus laevis extracts capable of nuclear assembly and transport have been employed. Using this system, it was demonstrated that the O-linked N-acetylglucosamine moiety of nuclear pore glycoproteins can be modified without altering nuclear transport. These extracts have also been used to demonstrate that the glycosylation and phosphorylation of nuclear pore glycoproteins change during the cell cycle. The findings suggest that there may be a coordinate regulation of glycosylation and phosphorylation which accompany the breakdown and reassembly of the nuclear pore during the cell cycle.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17009-08 LBM

## PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Tissue Specific and Hormone Regulated Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Hennighausen Research Chemist LBM, NIDDK

Others: R.A. McKnight Staff Fellow LBM, NIDDK  
U. Tillmann Special Volunteer LBM, NIDDK

## COOPERATING UNITS (if any)

USDA (R. Wall, V. Pursell); Univ. of Maryland (P.A. Furth);  
NCI (G. Smith); Glenn Merlino (NCI); Max-Planck-Institute, Gottingen, Germany  
(Peter Gruss); Zentrum for Molecular Biology, Heidelberg, Germany (Herman Bujard)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Developmental Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

3.0

## PROFESSIONAL

3.0

## OTHER

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Development and differentiation of the mammary gland during puberty, pregnancy and lactation appears to be controlled by hormones and growth modulators. We have previously shown that an endogenous milk protein, the whey acidic protein (WAP), can participate in mammary development. Temporal deregulated expression in mammary tissue of transgenic mice resulted in abrogated mammary development. Ectopic expression of the WAP gene under the control of the MMTV-LTR resulted in impaired mammary development as well as neoplasias in the coagulation gland. No growth aberrations were observed in other expressing tissues, such as the salivary gland. This suggests that WAP exerts its growth modulatory effects in a cell-specific manner.

The role of a given protein in tissue formation and differentiation processes can be evaluated using gain of function experiments in transgenic animals. However, the systems currently do not permit a spatial and temporal controlled activation and inactivation of transgenes. We established such a system using viral and bacterial tools. A binary system was built in which gene activity can be modulated using tetracycline. One line of mice carries a transgene composed of the human cytomegalovirus (HCMV) enhancer and the coding region for the DNA binding domain of the tetracycline repressor and the transcriptional activation domain VP16 from the Herpes simplex virus. The other line of transgenic mice carries a reporter gene posed of tetracycline operators next to a minimal promoter and the luciferase gene. Upon breeding, double transgenic mice have an activated reporter gene. Activity of this reporter gene should be subject to modulation by tetracycline as is currently investigated.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17024-10 LBM

PERIOD COVERED October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Inherited Disorders of Lysosomal Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. Myerowitz Research Chemist LBM, NIDDK

Others: J. Tropea IRTA LBM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Inherited Metabolic Disease

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

N-acetyl-neuraminidase is a lysosomal enzyme deficient in the inherited lysosomal storage disorders, sialidosis and galactosialidosis. Both diseases are clinically heterogeneous exhibiting both mild and severe forms. The former disease is thought to arise from mutations in the structural gene coding for neuraminidase. The latter disease results from a defect in a glycoprotein called protective protein which appears essential for maintenance of activity of both neuraminidase and  $\beta$ -galactosidase. All three proteins copurify and are believed to exist as a functional complex within the lysosome. Three approaches are being followed to clone the mammalian neuraminidase. (1) The complex has been purified from bovine testicular tissue for use in isolation of neuraminidase to obtain amino acid sequence information for cloning purposes. (2) A set of degenerate primers based on a five amino acid sequence motif found in viral, bacterial and trypanosome neuraminidases has been used to PCR amplify mRNA from human fibroblasts in attempts to capture an authentic cDNA fragment coding for neuraminidase. (3) An 80,000 bp segment of DNA located on chromosome 6 in the class III gene region of the major histocompatibility complex believed to contain the gene for neuraminidase has been isolated and we are attempting to trap the neuraminidase exons for use in screening a cDNA library. This approach is based on the following; a) a study that describes a single patient exhibiting the clinical features of both sialidosis and congenital adrenal hyperplasia along with the corresponding enzyme deficiencies of neuraminidase and 21 hydroxylase thereby suggesting a location for the neuraminidase gene proximal to that of the 21 hydroxylase gene on chromosome 6 in the class III gene region of the major histocompatibility complex and b) a suggestion of the presence of the sequence motif found in viral, bacterial and trypanosome neuraminidase in a newly discovered cluster of genes neighboring the 21 hydroxylase gene.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18007-14 LBM

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E.F. Grollman Medical Officer (Research) LBM, NIDDK

Others: A. Fanelli IRTA Fellow LBM, NIDDK

COOPERATING UNITS (if any)

LBM, NIDDK (G. Ashwell, A.R. Robbins); Walter Reed (S. Aloj); Roswell Park Cancer Inst. (J.YT Lau); Ito Hospital (K. Tahara).

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.65

PROFESSIONAL:

1.40

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The work continues to emphasize iodide transport in thyroid and the relationship of thyroglobulin sialylation and iodination necessary for normal thyroid hormone formation. The project has developed further in the cloning of iodide transport proteins from thyroid. For the project, a cDNA expression library, prepared from cultured rat thyroid cells superinduced to actively transport iodide, is being used. The screening assay uses a replica plate assay to detect iodide transport in a mutant line of rat thyroid cells that responds to thyrotropin demonstrated by elevations in cyclic AMP levels, but does not transport iodide. The particular methods for cloning were selected because of the lack of success using more traditional methods for cloning. Previous selection of iodide transport clones in this laboratory were based on the property of stilbene binding, a property associated with iodide loss from the thyroid into the follicular lumen. These clones are related to non-erythroid band 3 proteins. If the reconstitution of both types of iodide carriers (a sodium/iodide symporter and an iodide efflux channel) is successful, studies of the properties of iodide transport in thyroid will be more productive.

Collaborative studies demonstrated the role of thyrotropin in sialylation of thyroglobulin in cultured rat thyroid cells. These studies are now extended to address the glycosylation of thyroglobulin prepared from thyroid tissue from patients with two different thyroid diseases. In Graves' disease where there is elevated thyroid hormone levels, thyroglobulin is found to be both hyper- and abnormally sialylated. This contrasts with thyroglobulin isolated from endemic goiter which is both hypo-iodinated and hypo-glycosylated. The findings continue to support a significant role for sialylation in thyroglobulin secretion and iodination leading to normal thyroid hormone formation.

U. S. DEPARTMENT OF AGRICULTURE  
BUREAU OF PLANT INDUSTRY

PLANT INDUSTRY  
BUREAU OF PLANT INDUSTRY  
WASHINGTON, D. C.

1914

1915

1916

1917

1918

1919

1920

1921

1922

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Cell Regulation by Hormones, Growth Factors, Autoantibodies, and Oncogenes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L.D. Kohn Medical Director, USPHS, and LBM, NIDDK  
 Chief, Sec on Cell Regulation

Others: T. Ban Visiting Fellow (5 mos) LBM, NIDDK  
 S. Koenig Visiting Fellow (4 mos) LBM, NIDDK  
 H. Shimura Visiting Fellow LBM, NIDDK  
 A. Hidaka Visiting Fellow LBM, NIDDK  
 C. Giuliani Visiting Fellow LBM, NIDDK  
 Y. Shimura Visiting Fellow (10 mos) LBM, NIDDK  
 M. Saji Special Volunteer (1 yr) LBM, NIDDK  
 G. Napolitano Special Volunteer (1 yr) LBM, NIDDK  
 F. Okajima Special Volunteer (5 mos) LBM, NIDDK

## COOPERATING UNITS (if any)

NCI (D. Singer, S. Kimura); U. Texas Galveston (B. Prabhakar); Baylor U., Houston (F. Ledley); U. MD (W.A. Valente); U. Naples, Italy (R. DiLauro, E. Avvedimento, S. Aloj, & E. Consiglio); U. Udine, Italy (F.S. Ambesi-Impionbato); Tokyo Women's U, Tokyo, Japan (O. Isozaki); U. of Kyoto, Kyoto, Japan (T. Akamizu, T. Mori); French CNR, Paris, France (J. Chazotte).

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Cell Regulation

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

9.0

## PROFESSIONAL:

8.0

## OTHER:

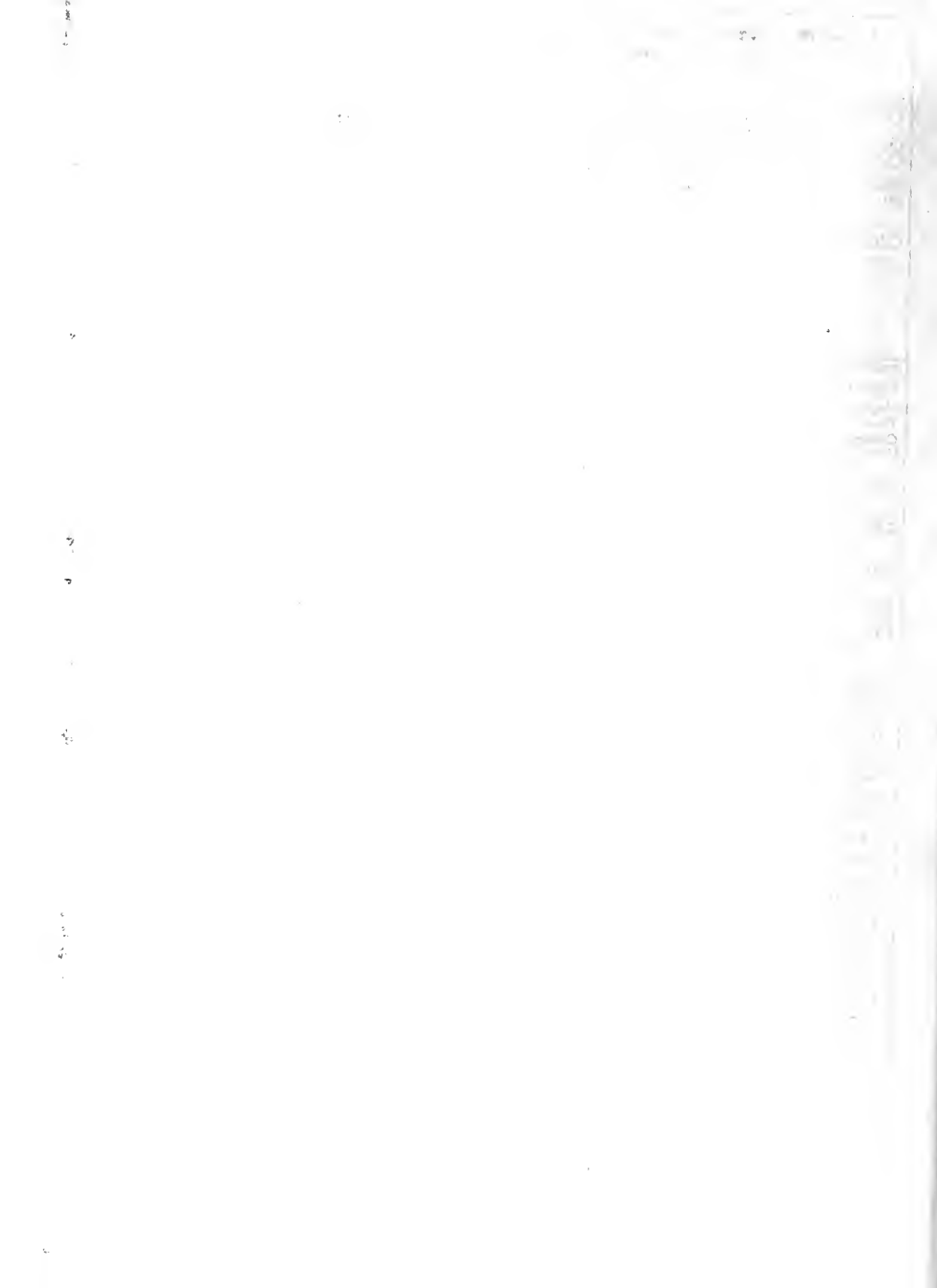
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## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

This laboratory is studying the structure-function relationships of the thyrotropin (TSH) receptor, the involvement of this receptor in autoimmune thyroid disease, as well as the relationship between thyroid autoimmune diseases and other organ-specific autoimmune diseases, i.e. Lupus or diabetes. We compare structure/ function of the TSH receptor to other glycoprotein hormone receptors and evaluate the interdependent regulation of thyroid function and growth by the TSH receptor and receptors for other ligands: gonadotropins, adrenergic, cholinergic, insulin, insulin-like growth factors (I and II), fibroblast growth factors, hydrocortisone, thyroid hormones, purinergic, bacterial toxins (cholera, pertussis, tetanus), interferon, and cytokines. Particular attention is given to identifying determinants on the receptors important for TSH and receptor autoantibody binding and signal transduction, as well as the transcriptional and posttranscriptional mechanisms by which TSH and the other receptors affect gene expression in thyroid cells. The relationship between oncogene transformation (thyroid tumors and adenomas), the development of autoimmunity, and the loss of normal regulation of thyroid function and growth is investigated. The role of different signal transduction mechanisms - cAMP, Ca/phosphoinositide and arachidonate - in thyroid cell growth and differentiated function is studied. We evaluate the role of membrane lipids in regulation of TSH receptor expression, LDL receptor expression, and cholesterol biosynthesis. We study the role of major histocompatibility antigens in the development of autoimmune thyroid diseases and organ specific immune diseases. Under development are human thyroid and islet cells which can grow in continuous culture, act as models of endocrine and thyroid disorders, and serve as donor cells for transplantation or gene therapy situations. These studies combine a molecular biology, cell biology and monoclonal antibody approach.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18009-14 LBM

## PERIOD COVERED

October 1, 1992 through September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Endocytosis, Secretion and Compartmentalization in Mutant CHO Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. R. Robbins Research Geneticist LBM, NIDDK

Others: C. W. Hall Research Chemist LBM, NIDDK

## COOPERATING UNITS (if any)

Office of Naval Research, Dept. of Defense (Dr. Constance Oliver)  
Dept. of Biochemistry, School of Public Health and Hygiene, Johns Hopkins University  
(Prof Sharon S. Krag)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

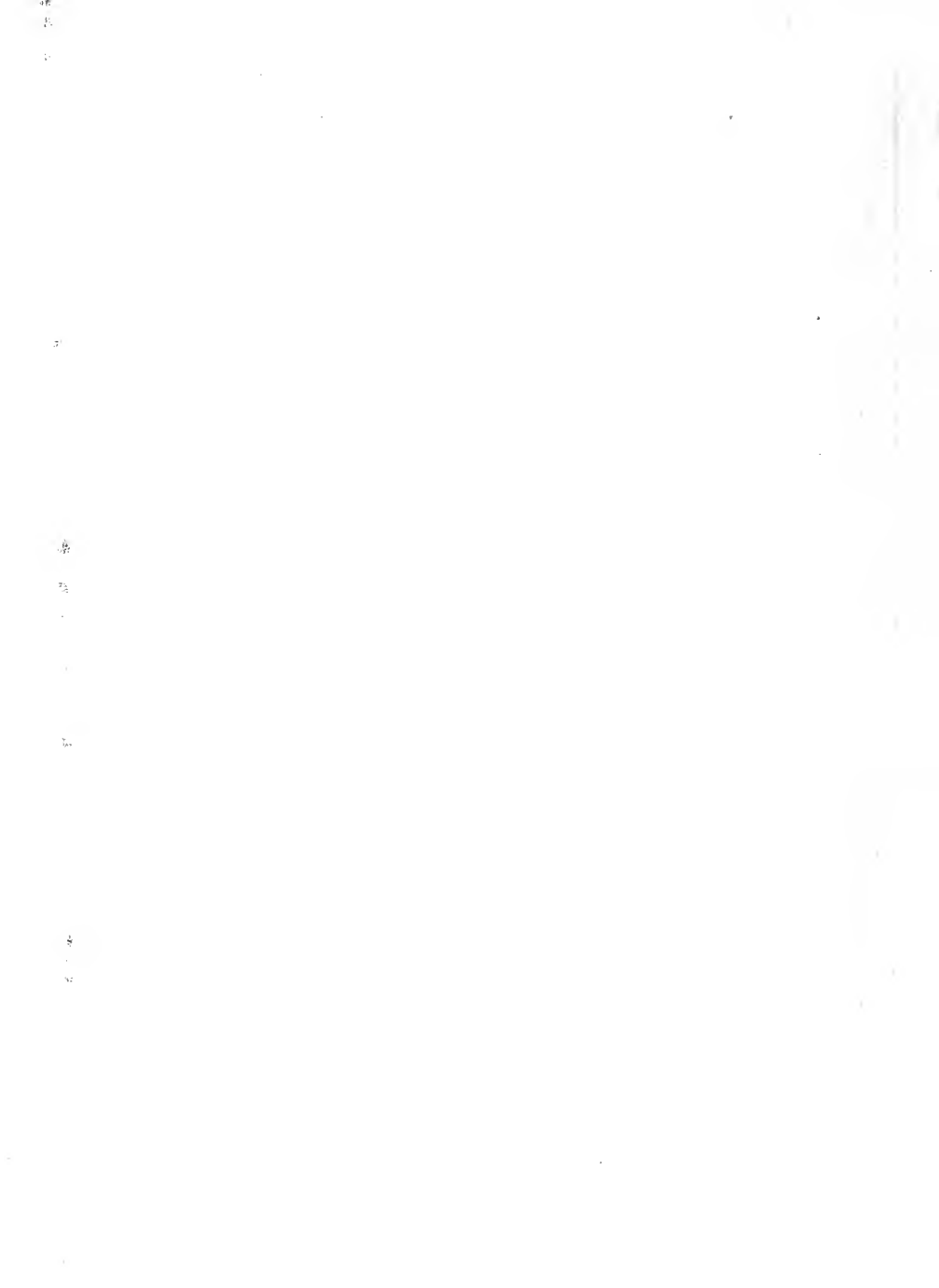
## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Association of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with other macromolecules and cellular structures (e.g. Band 3, microtubules, triad junctions) has been frequently reported. The functional significance, if any, of these associations is unknown. A single amino acid change in the GAPDH of a mutant Chinese hamster ovary cell (FD1.3.25) results in an association of that protein with microtubules that cannot be disrupted by agents (e.g. high salt, ATP) that effect dissociation of normal GAPDH; this correlates with genetically dominant alterations in endocytosis and aberrant association of late endosomes with microtubules observed in FD1.3.25. FD1.3.25 is a heterozygote, with normal and mutated GAPDH alleles; based on steady-state RNA levels, the mutated form represents 25% of the total GAPDH; moreover, the enzyme is a tetramer; thus very little of the GAPDH from FD1.3.25 is of "purely mutant" form. In order to study the properties of the mutant enzyme, GAPDH cDNA bearing the FD1.3.25 mutation has been overexpressed in E. coli.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18010-06 LBM

## PERIOD COVERED

October 1, 1992 through September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of Intracellular Traffic in HIV Infection

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J.A. Hanover Research Chemist LBM, NIDDK

Others:

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Cell Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS

0.5

## PROFESSIONAL

0.5

## OTHER

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Transport across the nuclear membrane is necessary during the life cycle of HIV. Once in the cytoplasm, HIV RNA is converted to double stranded DNA which must enter the nucleus. Viral regulatory proteins enter the nucleus; viral transcripts are exported into the cytoplasm. The viral REV and TAT proteins have been identified as key regulators of the transcription and transport of HIV envelope mRNA. These proteins contain sequences which target them to the nucleolus. Recently, it has been demonstrated that a reduction in cellular GTP levels induced by inhibitors of IMP dehydrogenase reduce the nucleolar accumulation of other nucleolar proteins. These inhibitors, which include ribavirin or mycophenolic acid, are being tested for their ability to interfere with HIV TAT and REV nucleolar localization. A means has also been devised for generating nuclei *in vitro* around exogenously added DNA. The method uses extracts from *Xenopus laevis* eggs. The nuclei assembled in such extracts mimic interphase nuclei in many ways and carry out active nuclear transport. These reformed nuclei have structures resembling nucleoli (termed pre-nucleoli). These preparations should allow the mechanism of REV action and the movement of other molecules involved in the HIV life cycle to be examined *in vitro*. In other studies the structure of the nuclear pore has been examined. The nuclear pore requires glycoprotein components for proper morphology and function.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 18012-09 LBM

PERIOD COVERED  
October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Direct Measurement of Forces between Membranes or Macromolecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V.A. Parsegian Guest Researcher LBM, NIDDK  
D.C. Rau Research Chemist LBM, NIDDK  
Others: S. Leikin Guest Researcher LBM, NIDDK

COOPERATING UNITS (if any) Brock Univ., Ontario, Canada (Dr. R.P.Rand); Univ. of Minn., Minneapolis, MN (Dr. D.F. Evans); Univ. of the Pacific, San Francisco, CA (Dr. J.A.Cohen); NHLBI (Dr. K.Gawrisch); NIAMS (Dr. N.L.Gershfeld); Josef Stefan Institute, Ljubljana, Slovenia (Dr. R.Podgornik); Free Univ., Berlin, Germany (Dr. M.M.Kozlov); Scientific Center, KFA, Juelich, Germany (Dr. A.A.Kornyshev); Univ. of British Columbia, Vancouver, Canada (Dr. E. Evans)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Enzymes and Cellular Biochemistry Section/Biophysics Unit

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The ability to measure directly the forces between membranes or between macromolecules is creating a new logic for thinking about molecular recognition, and folding. The outstanding feature of interaction is that as molecules or membranes approach contact, the important work of approach involves removal of organized water solvent from the apposing surfaces. These "hydration forces" are now recognized to act in materials as diverse as lipid bilayers, proteins, DNA double helices, and stiff polysaccharides.

During the current year we have measured intermolecular forces in native and reconstituted collagen fibers at various temperatures, pH, ionic conditions, and in the presence of several small solutes.

It has been shown that salt does not fully penetrate into the space between collagen triple helices. Osmotic pressure applied from outside by the excluded salt is an important component of collagen fiber assembly.

Force measurements have demonstrated that temperature-favored assembly of the fibers is driven by water-mediated hydrogen bonding between the apposing polar residues rather than by the hydrophobic effect, usually invoked to explain assembly of proteins. One can now think of a competition between repulsive and attractive hydration forces, depending on how well protein surfaces match each other.

A dependence of hydration forces between DNA molecules on small solutes present in the solution has been studied.

An unusual re-entrant liquid-gel-liquid phase transition sequence, observed during measurement of forces between didodecylphosphate bilayers, has been explained.

An osmotic stress technique for measuring forces between spherical particles has been developed.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18013-06 LBM

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Physics of Ionic Channels and other Proteins with Aqueous Cavities

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V.A. Parsegian Guest Researcher LBM, NIDDK  
S. M. Bezrukov Visiting Scientist LBM, NIDDK

Others: J.J. Kasianowicz Guest Researcher LBM, NIDDK  
D.C. Rau Research Chemist LBM, NIDDK  
R. Brutyan Special Volunteer LBM, NIDDK  
S. Keller Special Volunteer LBM, NIDDK

COOPERATING UNITS (if any)

LPTB, NICHD (Dr. J.J. Zimmerberg); John Hopkins Univ., Baltimore, MD (Dr. A.Harris); Office of Naval Research (Dr. I.Vodyanov); Princeton Univ., Princeton, NJ (Drs. S.M.Gruner, M.W.Tate); Univ. of Maryland, College Park, MD (Dr. M.Colombini); Monell Chemical Senses Center, Philadelphia, PA (Dr. A.M.Feigin)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Enzymes and Cellular Biochemistry Section/Biophysics Unit

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

6.0

PROFESSIONAL:

6.0

OTHER:

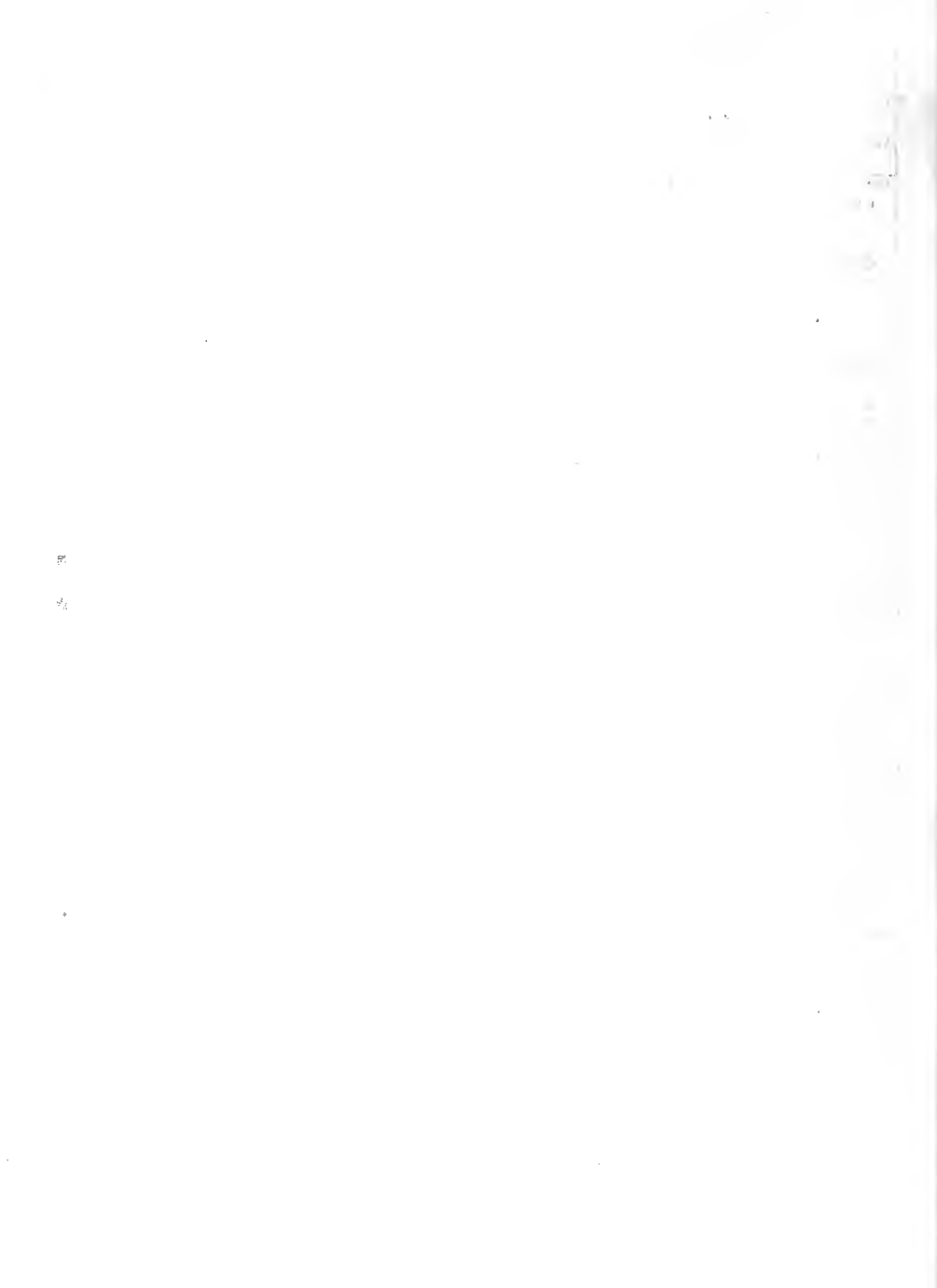
CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

The kinetics of *S. Aureus* alpha-toxin channels were examined by "noise" analysis at several different pH's to measure the kinetics of proton binding to ionizable sites within the channel. Recognizing that this binding follows first-order kinetics, and noting that proton binding creates a step change in channel conductance one sees that the responsible ionizable sites have a pK of 5.5 and that the association and dissociation rate constants are  $8 \times 10^9$  and  $10^5$  respectively. These values imply that the ionizable residues are freely accessible to the aqueous phase and that the active groups are either histidines, glutamic acids or aspartic acids.

By exposing ionic channels to neutral polyethyleneglycols of different molecular weight, we have been able to generalize the use of water-soluble polymers to probe channel structure. Large excluded solutes in membrane-bathing solutions create an osmotic work of transition between different conductance states, a work proportional to the difference in polymer-inaccessible aqueous spaces of these states. Progressively smaller polymers begin to penetrate the channel space, to cause a decrease in channel conductance and to exert a correspondingly reduced effective osmotic pressure. We find that the channel, a kind of ultimate molecular sieve, appears to exclude polymers on the basis of their rotational volume calculated from radius of gyration (rather than the polymer cross-section or diameter typically used to gauge channel size). In a particular case of alamethicin, polymer probing reveals a fixed (about  $3,000 \text{ \AA}^3$ ) aqueous volume change with each successive step between the five different conductance states. These states, then, are collections of conducting units of nearly the same conductance rather than pores of successively larger radius.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18014-09 LBM

## PERIOD COVERED

October 1, 1992 through September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Physical Properties of DNA and DNA-Protein Complexes

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D.C. Rau Research Chemist LBM, NIDDK

Others: N. Yu. Sidorova Visiting Fellow LBM, NIDDK

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## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

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## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Changes in water binding upon complex formation have been measured for several specific sequence DNA binding proteins. Large numbers of water molecules are displaced in the binding reaction: ~ 100 for the binding of the galactose operon repressor, ~ 50 for cAMP receptor protein (CAP), and ~ 30-40 for the specific binding of the restriction nuclease Eco RI. These changes in hydration correlate approximately with the size of the DNA recognition sequence. In addition, the binding of a second galactose operon repressor to the naturally occurring 2 operator DNA fragment shows an additional release of water due to protein-protein interactions.

Changes in hydration accompanying the binding of analogs of the antibiotic netropsin to specific DNA sequences are being measured. A correlation between water release and binding strength to different sequences has now been observed. The complete set of data of water release and binding energy for many different sequences will allow a quantitative analysis of the importance of the hydration forces observed in condensed systems for the specific binding of peptides and proteins to DNA in dilute solution.

The electric birefringence of Acanthamoeba myosin II filaments has revealed a large change in LMM-HMM junction flexibility with ATP binding to myosin heads. In addition to the well established crossbridge motions of myosin heads, a concomitant change in filament stiffness coupled to ATP binding and hydrolysis appears involved in the force generation cycle of myosin.

THE UNIVERSITY OF CHICAGO

## ANNUAL REPORT

### THE LABORATORY OF CELL BIOLOGY AND GENETICS

#### NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory of Cell Biology and Genetics carries out a broad program of investigation into exocrine and endocrine secretion and the molecular events regulating these processes. The specific systems studied include chromaffin cells from adrenal medulla and brain, beta cells in islets of Langerhans, gonadotropes from brain, and various mesodermal cells. The Laboratory also pursues applications of these basic studies to clinical problems including cystic fibrosis, Parkinson's disease, Alzheimer's disease, and vitamin deficiency states. The following studies have been performed in pursuit of these goals.

#### I. REGULATION OF SECRETION FROM CHROMAFFIN CELLS

##### A. Catecholamine secretion induced by tetraethylammonium from cultured bovine adrenal chromaffin cells

The resting potential in adrenal medullary chromaffin cells is maintained by the activity of different  $K^{2+}$ -channels. Blockade of  $K^{2+}$ -channels should, at least in principle, lead to membrane depolarization, and the ensuring activation of voltage-gated  $Ca^{2+}$ -channels should promote  $Ca^{2+}$  entry and catecholamine (CA) secretion. In support of this mechanism we found that the  $K^{2+}$ -blocker tetraethylammonium (TEA) depolarized the chromaffin cell membrane, induced a substantial elevation in cytosolic  $[Ca^{2+}]$ , and a dose-dependent CA secretion reaching a maximum at 50 mM of approx. 10% of the total CA in the cells. In addition, TEA-induced CA secretion was found to be absolutely dependent on  $[Ca^{2+}]_o$ . In the presence of  $[Ca^{2+}]_o$ , TEA-stimulated CA release was blocked completely by elevated  $[MgCl_2]_o$  (12 mM), and inhibited in part by the  $Ca^{2+}$ -channel antagonist nifedipine. The  $Ca^{2+}$ -channels agonist Bay K-8644 markedly enhanced TEA-evoked CA release suggesting the involvement of L-type  $Ca^{2+}$ -channels. Since, external application of TEA (30-50 mM) markedly blocked outward  $K^{2+}$  currents but not inward currents carried by  $Na^{2+}$  and  $Ca^{2+}$ , we conclude that TEA stimulates CA secretion by blocking those  $K^{2+}$ -channels involved in the maintenance of the resting membrane potential.



B. Strychnine inhibits nicotinic acetylcholine receptor activation in bovine adrenal chromaffin cells

Strychnine is known as a potent and selective antagonist of the inhibitory glycine receptor in the central nervous system. The strychnine binding site on the glycine receptor is located on a 48 kDa domain, which shares homology with the nicotinic acetylcholine receptor polypeptide family. We found that strychnine (1-100  $\mu$ M) inhibits nicotine- and oxotremorine-M-stimulated catecholamine secretion, but not  $K^{2+}$ -, histamine- or angiotensin II-induced secretin from bovine chromaffin cells in a reversible manner. Strychnine also inhibits the nicotine-induced elevation in membrane potential and intracellular  $Ca^{2+}$  concentration. Strychnine appears to compete with nicotine in its effect, but does not modify the apparent positive cooperativity of the nicotine binding sites ( $n_H=2.55$ ). These results suggest that strychnine interacts with the agonist binding site of the nicotinic acetylcholine receptor expressed by the chromaffin cell.

C. Identification and Purification of A 28Kda Protein From Bovine Chromaffin Cell Cytosol Which Reversibly Interacts With Lipid Phase of The Membrane: Possible Role in Fusion and Exocytosis

Cytosolic proteins in the chromaffin cell may play a vital role in membrane fusion processes during granule assembly and secretion, and we have examined this cell for the presence of amphipathic proteins able to transiently penetrate the lipid phase of the membrane. Our experimental approach involved using the lipophilic probe [125]-5-iodonaphtyl-1-azide (125INA). Cultured bovine chromaffin cells labelled with 125INA were subjected to PAGE and autoradiography and analyzed for the presence of radiolabelled cytosolic and membrane-bound proteins. The cytosol contained only one labelled protein of molecular weight of 28Kda (p28K) which we have identified on the basis of partial microsequencing. Sequence analysis showed that p28k has high (>85%) homology with two other proteins previously purified from bovine brain cytosol and rat epididymal fluid. Among the different cell types tested (HeLa, NIH3T3, CFPAC, SKNSH) only rat mast cells (RBL) showed a similar pattern of labelling of the cytoplasmic protein. Chromaffin granules, as well as whole chromaffin cells, supported the labelling of p28K. In addition the penetration of p28K into the hydrocarbon core of the lipid bilayer was confirmed by energy transfer labelling from a non-exchangable fluorescent membrane bilayer probe. We take these data to indicate that p28k may play a role in regulation of membrane interactions within chromaffin cell including fusion events associated



with the exocytotic process.

D. Aggregation and fusion of chromaffin granules and liposomes by lipocortin I (DES 1-12)

Annexins are a family of proteins that share the ability to associate with membrane phospholipids in a  $\text{Ca}^{2+}$ -dependent manner. We have isolated and purified three water-soluble forms of bovine lipocortin I (annexin I): intact, DES 1-12, and DES 1-26. Aggregation of chromaffin granules by lipocortin I was activated by both  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$ . The most active form was found to be the DES 1-12 which had previously been shown to cause fusion of chromaffin granules in the presence of  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$  and 10  $\mu\text{M}$  arachidonic acid. We report here that DES 1-12 induced membrane mixing and aggregation of PS extruded liposomes. Fusion was induced by DES 1-12 but not by intact lipocortin I. Moreover, addition of synthetic peptide with the sequence of the first 12 a.a. of lipocortin I blunted the fusogenic effect of DES 1-12. Vesicle aggregation and fusion were dependent on the concentration of divalent cations with  $K_{1/2}$  of 270  $\mu\text{M}$  for  $\text{Ca}^{2+}$  and 170  $\mu\text{M}$  for  $\text{Ba}^{2+}$ , and had a sigmoidal curve typical of a positively cooperative reaction.  $\text{Mg}^{2+}$ -induced fusion required much higher concentration (2.5 mM) and the activation was linear with respect to  $[\text{Mg}^{2+}]$ . Aggregation and fusion rates were enhanced by replacement of salt with sucrose and by lowering the pH. We conclude that (a) lipocortin I (DES 1-12) can mediate aggregation and fusion in the presence of  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$ ; (b) the fusion process, at least in the vesicles, is apparently cooperative; (c) in bovine lipocortin I, fusion depends on the truncation of the first 12 amino acids on the N-terminal domain.

E. Annexins In The Chromaffin Cell: Immunolocalization and Quantification in Resting and Stimulated Cells

The annexins are a family of proteins, whose functions are yet to be fully elucidated. Based on their ability to aggregate chromaffin granules in the presence of  $\text{Ca}^{2+}$ , it has been proposed that the annexins, particularly synexin (annexin VII) and calpactin (annexin II), play a role in  $\text{Ca}^{2+}$ -mediated exocytotic secretion. We studied the localization of four annexins (annexins I, II, V and III) in resting and actively secreting bovine adrenal chromaffin cells. Cells were isolated and cultured for three days, stimulated with nicotine (10  $\mu\text{M}$ ) or  $\text{BaCl}_2$  for 2 minutes, and embedded in LR white. Ultrathin sections were incubated with polyclonal annexin antibodies and immunogold, and examined in the electron microscope. A random series of micrographs of epinephrine cells containing granule, nuclear and/or plasma membrane areas were taken, and the gold particle densities in these areas were determined. All annexins were present in the three





were determined. All annexins were present in the three examined cell compartments, albeit in different proportions. Annexins were also localized to endoplasmic reticulum and mitochondria. Significant changes in the gold particle density for the various annexins were detected upon secretory stimulation, indicating annexin concentration or conformational changes. Our results are consistent with the hypothesis that the annexins are involved in the exocytotic secretory process in chromaffin cells.

**F. Effect of MPTP on Dopaminergic Neurons in the Goldfish Brain: A Light and Electron Microscopic Study**

The neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine) causes a Parkinsonian syndrome in humans and other primates by a selective destruction of dopaminergic neurons after its conversion to MPP<sup>+</sup> by monamine oxidase B (MAO-B) in non-neuronal cells. When goldfish (*Carassius auratus*) are injected with MPTP (50 mg/kg), they also transiently show a Parkinsonian-like behaviour, and their forebrain and midbrain content of dopamine and norepinephrine is substantially decreased (Pollard et al, FASEB J. 6:3108-3116, 1992). The MAO-B inhibitor L-deprenyl (mg/Kg) protects goldfish against all toxic effects of MPTP. In an effort to delineate the specific neurons targeted by MPTP, we have investigated the effect of MPTP on the distribution and ultrastructure of dopaminergic neurons in the goldfish brain by light and electron microscopy: Brains of goldfish treated with MPTP and/or L-deprenyl for three days were fixed and cut into 50µm sections on a vibratome, and subsequently processed for EM or immunocytochemistry, using an antibody against tyrosine hydroxylase (TH) as a marker for dopaminergic neurons, which we imaged by the diaminobenzidine method (DAB). We found TH-positive neurons in the following brain areas: (A) Telencephalon: 1. Olfactory bulb; 2. Nucleus (N.) olfactorius medialis; 3. N. preopticus; 4. N. telencephali pars medialis; 5. N. suprachiasmaticus, (B) Diencephalon: 6. N. dorsolateralis thalami; 7. and 8. N. of anterior and posterior paraventricular organ, respectively; 9. N. posterior tuberis, (C) Tegmentum: 10. N. of isthmus tegmentum, (D) Vagal lobe: 11. N. of area postrema. Following MPTP treatment, we observed a decrease in the amount and staining intensity of TH-positive neurons, as well as a more diffuse staining pattern, mainly in area #4, and areas #7 and # around the third ventricle. Electron microscopy confirmed that neuronal damage occurred in these regions. In area #4, L-deprenyl appeared to prevent these degenerative changes. Ultrastructurally, MPTP treatment caused swelling of dendrites and cell death in brain areas containing dopaminergic neurons. In addition, L-deprenyl by itself caused damage in the subepithelial layers surrounding the ventricles in both



activated by caffeine. The caffeine-activated  $\text{Ca}^{2+}$ -channel known to be present in SR membranes from vertebrate skeletal muscle is, in our opinion, the most probable site of action for tetracaine.

B. Apamin-sensitive potassium channels mediate agonist-induced oscillations of membrane potential in pituitary gonadotrophs

In cultured rat pituitary gonadotrophs, gonadotropin-releasing hormone (GnRH) induces rapid hyperpolarization of the cell membranes and causes cessation of the spontaneous electrical activity present in non-stimulated cells. This initial response to GnRH is followed by slow oscillations of membrane potential  $V_m$  which often exhibit brief bursts of action potentials (AP) fired from the peak of the oscillations. The hyperpolarization waves are synchronous with GnRH-induced elevations of cytoplasmic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ), such that  $V_m$  maxima alternate with the peak values of  $[\text{Ca}^{2+}]_i$ . The  $V_m$  oscillations result from repetitive activation of apamin-sensitive  $\text{K}^+$  channels by cytoplasmic  $\text{Ca}^{2+}$ . Thus, GnRH activation of  $\text{Ca}^{2+}$  mobilization can generate a bursting pattern of membrane potential through the activation of  $\text{K}^+$  channels against a background of spontaneous electrical activity.

C. Calcium Signaling and Secretory Responses In Agonist-Stimulated Pituitary Gonadotrophs

In cultured pituitary gonadotrophs, gonadotropin-releasing hormone (GnRH) caused dose-dependent and biphasic increases in cytoplasmic calcium concentration ( $[\text{Ca}^{2+}]_i$ ) and LH release. Both extra- and intracellular calcium pools participate in GnRH-induced elevation of  $[\text{Ca}^{2+}]_i$  and LH secretion. The spike phase of the  $[\text{Ca}^{2+}]_i$  response represents the primary signal derived predominantly from the rapid mobilization of intracellular  $\text{Ca}^{2+}$ . In contrast, the prolonged phase of the  $\text{Ca}^{2+}$  signal depends exclusively on  $\text{Ca}^{2+}$  entry from the extracellular pool. The influx of  $\text{Ca}^{2+}$  occurs partially through dihydropyridine-sensitive calcium channels. Both  $[\text{Ca}^{2+}]_i$  and LH responses to increasing concentrations of GnRH occur over very similar time scales, suggesting that increasing degrees of receptor occupancy are transduced into amplitude-modulated  $\text{Ca}^{2+}$  responses, which in turn activate exocytosis in a linear manner. However, several lines of evidence indicated the complexity over the relationship between  $\text{Ca}^{2+}$  signaling and LH exocytosis. In contrast to  $[\text{Ca}^{2+}]_i$  measurements in cell suspension, single cell  $\text{Ca}^{2+}$  measurements revealed the existence of a more complicated pattern of  $\text{Ca}^{2+}$  response to GnRH, with a biphasic response to high agonist doses and prominent oscillatory responses to lower GnRH concentrations, with a log-linear correlation between GnRH dose and the frequency of  $\text{Ca}^{2+}$  spiking. In addition, analysis of the magnitudes



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of the  $[Ca^{2+}]_i$  and LH responses of gonadotrophs to a wide range of GnRH concentrations in the presence and absence of extracellular  $Ca^{2+}$ , and to  $K^+$  and phorbol ester stimulation, showed non-linearity between these parameters with amplification of  $[Ca^{2+}]_i$ -mediated exocytosis. Studies on cell depleted of protein kinase C under conditions that did not change the LH pool suggested the participation of protein kinase C in this amplification, especially during the plateau phase of the secretory response to GnRH.

#### D. Mechanism of Agonist-induced $[Ca^{2+}]_i$ Oscillations in Pituitary Gonadotrophs

Gonadotropin-releasing hormone (GnRH) activates oscillatory  $Ca^{2+}$  signaling in pituitary gonadotrophs at a frequency up to 25 min<sup>-1</sup> that is dose-dependent and is determined by the degree of receptor-mediated inositol 1,4,5-trisphosphate ( $Ins(1,4,5)P_3$ ) formation. Similar dose-dependent and frequency-modulated  $Ca^{2+}$  oscillations were elicited by intracellular administration of  $Ins(1,4,5)P_3$  and its nonhydrolyzable analogs consistent with models in which  $Ins(1,4,5)P_3$  levels determine the frequency of  $Ca^{2+}$  oscillations but do not fluctuate in synchrony with  $[Ca^{2+}]_i$ . At constant agonist concentrations,  $Ca^{2+}$  spiking varied in amplitude, with a number of progressively larger transients before the onset of maximal oscillations, followed by a gradual decrease in spike amplitude that was accompanied by an increase in spiking frequency. The decline in the amplitude and increase in frequency of  $Ca^{2+}$  transients during stimulation by GnRH were not related to a decrease in the propagation of the  $Ca^{2+}$  signal within the cell but were associated with gradual depletion of the agonist-sensitive  $Ca^{2+}$  pool. Once initiated, the pattern of  $Ca^{2+}$  spiking was not altered by blockade of receptor occupancy, by inhibition of phospholipase C, or by reduction of extracellular  $[Ca^{2+}]_o$ . also, the endoplasmic reticulum ( $Ca^{2+}$ )-ATPase blocker, thapsigargin, could substitute for  $Ins(1,4,5)P_3$  in initiating the oscillatory  $Ca^{2+}$  response. These findings indicate that although the  $Ins(2,4,5)P_3$  concentration determines the pattern of transients at the initiation of the oscillatory  $Ca^{2+}$  signal, maintenance of the signal does not require a sustained rise in  $Ins(1,4,5)P_3$ . Since the frequency of  $Ca^{2+}$  oscillations is also influenced by depletion of luminal  $[Ca^{2+}]_r$ , it is possible that the  $Ins(1,4,5)P_3$ -sensitive channels in the endoplasmic reticulum are tonically inhibited by high intraluminal  $Ca^{2+}$  levels and that  $Ins(1,4,5)P_3$  surmounts such inhibition by promoting  $Ca^{2+}$  discharge. When a critical level of  $Ca^{2+}$  discharge is attained, repetitive  $Ca^{2+}$  transients are generated by an autocatalytic mechanism in which a sustained rise in  $Ins(1,4,5)P_3$  is not an essential requirement.





### III. GLUCOSE CONTROL OF INSULIN SECRETION

#### A. Inter-islet Variability in Insulin Secretion Response To Glucose (G) and Potassium (K) in Single Microdissected Mouse Islets

The insulin secretion response of individual islets of Langerhans to an identical G stimulus were compared to the response to a standard depolarization induced by increasing K concentration. Individual islets were microdissected from the tail portion of the pancreas of normal mice, isolated in separate chambers, & perfused with a 5.6 mM G solution for >60 min to allow them to stabilize; then, 80 1-minute collections were taken with each islet being perfused with solutions of 5.6 mM G (10 min), 22 mM G (20 min), & 5.6 mM G (30 min), 30 mM K (10 min), & 5.6 mM G (10 min). Insulin in each collection & total insulin content of each islet measured. Large variability in the secretory response to 22 mM G was found among the islets, ranging from no response to 27-fold stimulation over basal. The variability among the responsive islets appeared to be related to islet insulin content but not to basal rate of insulin secretion. Variability was also observed in response to 30 mM K, but there was no correlation in the relative proportion of the G-stimulated & K-stimulated responses, except that islets that did not respond to G also did not respond to K. It is proposed that variability may reflect a qualitative difference among islets or a slow, dynamic cycling of islets between resting & hyperactive states. Furthermore, depolarization & calcium influx appear not to be the underlying determinants of islet response variability.

#### B. Glucose-Induced Lowering of Intracellular Calcium in Single Mouse Islets

Glucose has complex effects on the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) of pancreatic islets. While the steady-state effect of glucose is to induce rhythmic oscillations of  $[\text{Ca}^{2+}]_i$ , the earliest detectable effect of glucose (termed phase 0) is to cause a lowering of  $[\text{Ca}^{2+}]_i$ . We investigated this phenomenon in more detail in enzymatically-isolated C57BL/KsJ mouse islets loaded with the fluorescent indicator fura-2. In the presence of 2 mM glucose,  $[\text{Ca}^{2+}]_i$  was <100 nM. On exposure to 12 mM glucose, we observed a highly reproducible 30-50% decline in  $[\text{Ca}^{2+}]_i$ , which was sustained for approximately 2-3 min before  $[\text{Ca}^{2+}]_i$  began to rise. The  $[\text{Ca}^{2+}]_i$ -lowering effect of glucose was not reproduced by exposure to 5  $\mu\text{M}$  glyburide, indicating that the effect was unrelated to  $\text{K}^{\text{ATP}}$  channel blockade. Indeed, application of glucose after exposure to glyburide (which raised  $[\text{Ca}^{2+}]_i$  nearly 4-fold above basal values) resulted in a dramatic lowering of  $[\text{Ca}^{2+}]_i$  to resting levels, suggesting that the  $\text{Ca}^{2+}$ -lowering system is of large



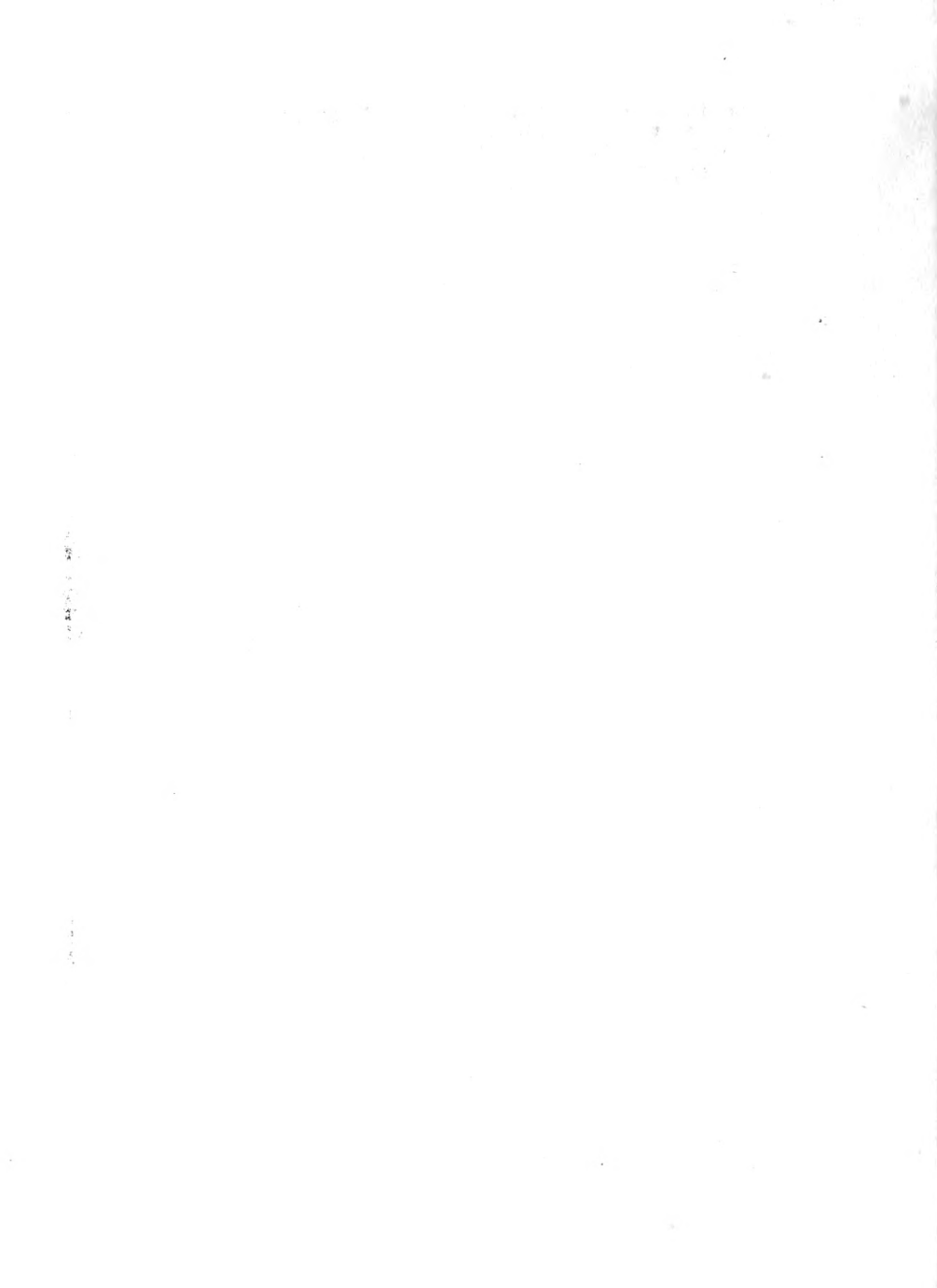
capacity. Application of 1  $\mu\text{M}$  thapsigargin, a specific antagonist of endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase activity, completely abolished the  $[\text{Ca}^{2+}]_i$ -lowering effects of glucose. Since it was likely that glucose was activating the pump by ATP generation secondary to glucose metabolism, we compared the effects of different concentrations of glucose on  $[\text{Ca}^{2+}]_i$ . An increase of glucose concentration from 2 to 4 mM was sufficient to cause the phase 0-type response, but was unable to induce subsequent  $[\text{Ca}^{2+}]_i$  oscillations, indicating that the  $\text{Ca}^{2+}$ -ATPase has a lower  $K_m$  for ATP than the  $K_{\text{ATP}}$  channel, whose closure initiates phasic  $[\text{Ca}^{2+}]_i$  oscillations. We conclude that the phase 0 effect of glucose depends upon activation of a high-affinity, high-capacity  $\text{Ca}^{2+}$ -ATPase located in the  $\delta$ -cell endoplasmic reticulum.

C. Expression of Yeast Hexokinase in Pancreatic  $\delta$  Cells of Transgenic Mice Reduces Blood Glucose, Enhances Insulin Secretion, and Decreases Diabetes

It has been proposed that endogenous hexokinases of the pancreatic  $\delta$  cell control the rate of glucose-stimulated insulin secretion and that genetic defects that reduce  $\delta$ -cell hexokinase activity may lead to diabetes. To test these hypotheses, we have produced transgenic mice that have a 2-fold increase in hexokinase activity specific to the pancreatic  $\delta$  cell. This increase was sufficient to significantly augment glucose-stimulated insulin secretion of isolated pancreatic islets, increase serum insulin levels in vivo, and lower the blood glucose levels of transgenic mice by 20-50% below control levels. Elevation of hexokinase activity also significantly reduced blood glucose levels of diabetic mice. These results confirm the role of  $\delta$ -cell hexokinase activity in the regulation of insulin secretion and glucose homeostasis. They also provide strong support for the proposal that reductions in  $\delta$ -cell hexokinase activity can produce diabetes.

D. Ascorbic Acid in Pancreatic Beta Cells and Regulation of Membrane Potential

Ascorbic acid (vitamin C) was discovered in normal pancreatic beta cells in mM concentration, was predominantly cytosolic, and was depleted >95% as were cultured. When 100  $\mu\text{M}$  ascorbic acid was added to the culture medium, a 40 fold intracellular concentration gradient was maintained. We also investigated the effect of extracellular ascorbic acid on beta cell membrane potential. Within 20 seconds, external ascorbic acid at >100  $\mu\text{M}$  suppressed glucose-induced depolarization of beta cells. Suppression was concentration dependent, reversible within 20 seconds, and specific to ascorbic acid and not other reducing agents nor the ascorbic acid oxidation product dehydroascorbic acid. Since ascorbic acid is



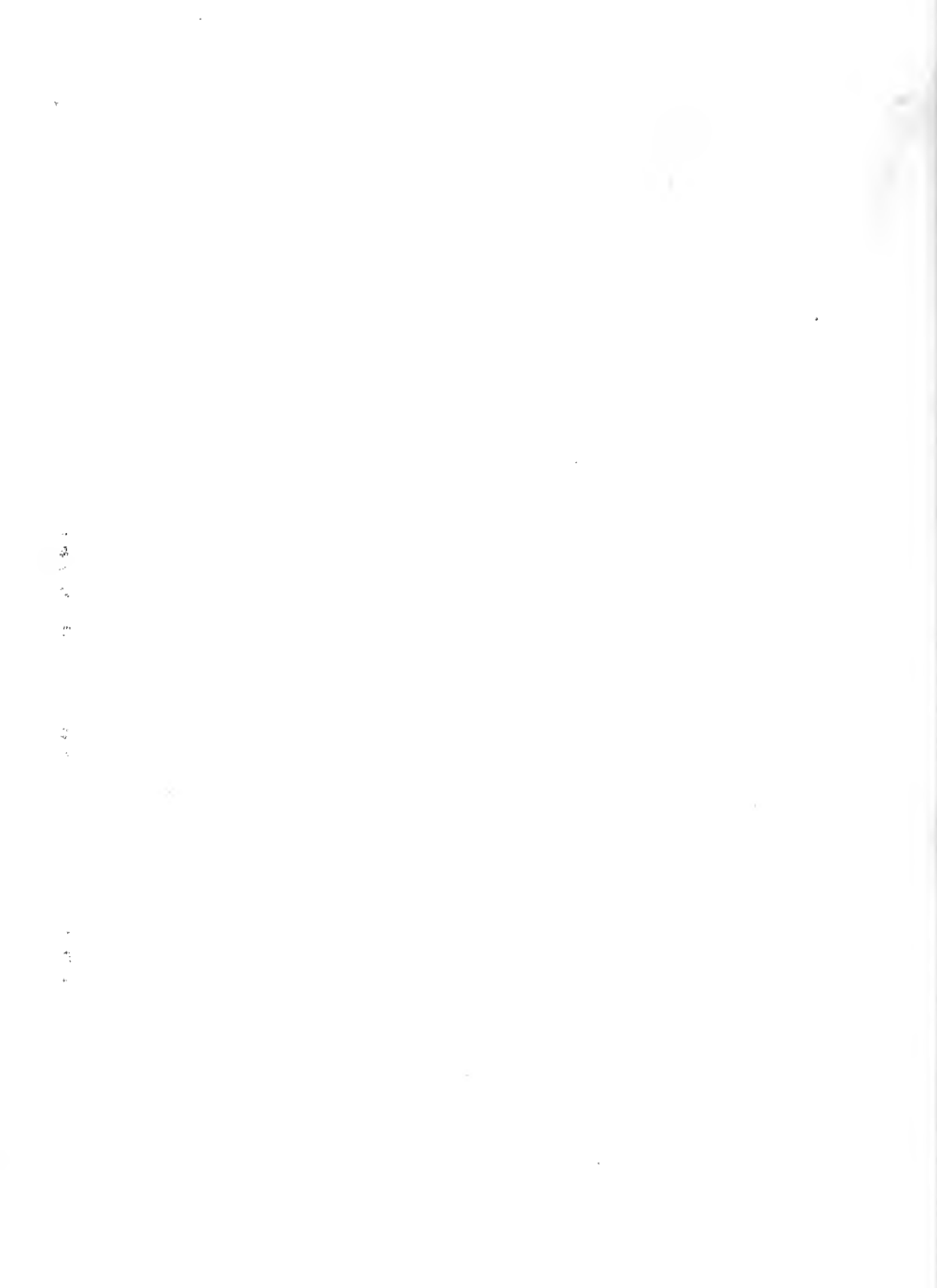
substantially accumulated by beta cells under different conditions, intracellular ascorbic acid may have an important physiologic function. Furthermore, since external ascorbic acid transiently inhibits beta cell depolarization, the vitamin might thereby modulate glucose sensing.

E. Prolactin Induces Maturation of Glucose Sensing Mechanisms In Cultured Neonatal Rat Islets

The effects of PRL treatment on insulin content and secretion, and  $^{86}\text{Rb}$  and  $^{45}\text{Ca}$  fluxes from neonatal rat islets maintained in culture for 7-9 days were studied. PRL treatment enhanced islet insulin content by 40% and enhanced early insulin secretion evoked by 16.7 mM glucose. Insulin release stimulated by oxotremorine-m, a muscarinic agonist, in the presence of glucose (8.3 or 16.7 mM) was unchanged by PRL treatment. However, PRL treatment potentiated phorbol 12,13-dibutyrate-stimulated insulin secretion in the presence of the above glucose concentrations. PRL treatment potentiated the reduction in  $^{86}\text{Rb}$  efflux induced by glucose or tolbutamide and enhanced the increase in  $^{86}\text{Rb}$  efflux evoked by diazoxide. PRL treatment slightly potentiated the increment in  $^{45}\text{Ca}$  uptake induced by high concentrations of  $\text{K}^+$ , but failed to affect the increment evoked by 16.7 mM glucose. Since glucose-induced  $^{45}\text{Ca}$  uptake was not affected by PRL, we suggest that the enhancement in first phase insulin secretion evoked by glucose in the PRL-treated islets occurs at a step in the secretory process that may involve protein kinase-C. These data further support observations that PRL treatment increases islet sensitivity to glucose.

F. Differences in  $\text{K}^{2+}$  Permeability Between Cultured Adult and Neonatal Rat Islets of Langerhans in Response to Glucose, Tolbutamide, Diazoxide, and Theophylline

The effects of glucose, tolbutamide, and diazoxide on  $\text{K}^{2+}$  permeability in neonatal and adult rat pancreatic islets, maintained in culture 1 week, were investigated by measuring  $^{86}\text{Rb}$  outflow rate from prelabeled islets. In the absence of glucose, the  $^{86}\text{Rb}$  efflux was significantly lower in neonatal than adult islets. Raising the glucose concentration to 2.8, 5.6, 8.3, and 11.1 mM produced a marked reduction in the  $^{86}\text{Rb}$  efflux in adult islets but only a minor reduction in neonatal islets. The effect of tolbutamide to reduce, and diazoxide to increase, the  $^{86}\text{Rb}$  efflux was also less in neonatal islets. These results are consistent with previously reported differences in insulin secretion from neonatal and adult islets in culture.



G. Elemental composition of secretory granules in pancreatic islets of Langerhans

We have characterized, by electron probe microanalysis, rapidly frozen cultured rat islets at the level of individual secretory granules. Elemental analysis of thin, dried cryosections showed that beta granules could be distinguished by high Zn, Ca, and S, whereas non-beta (mainly alpha) granules contained elevated P and Mg. Although a single granule type predominated in a particular cell, some rebel granules were found in A cells that had the compositional fingerprint of B cell granules. Zn, which was found in millimolar concentrations in B cell granules, was considered a marker for the insulin storage complex. The data indicate that non-B islet cells in the adult pancreas may produce insulin-containing organelles and that, when glucagon and insulin are co-expressed, these hormones are packaged in separate granules.

IV. CHLORIDE CHANNELS AND CYSTIC FIBROSIS

A. A<sub>1</sub>-Receptor Antagonist 8-Cyclopentyl-1,3-Dipropylxanthine (CPX) Selectively Activates Chloride Efflux From Human Epithelial and Mouse Fibroblast Cell Lines Expressing the CFTR (ΔF508) Mutation, but Not the Wild Type CFTR

Cystic fibrosis is an autosomal recessive disorder affecting chloride transport in pancreas, lung, and other tissues, which is caused by mutations in the cystic fibrosis transmembrane regulator (CFTR). The A<sub>1</sub>-receptor antagonist CPX (8-cyclopentyl-1,3 -dipropyl-xanthine) stimulates <sup>36</sup>[Cl-] efflux from pancreatic CFPAC-1 cells which bear the ΔF508 genotype common to most cases of cystic fibrosis (13, Eidelman, et al, 1992). By contrast, correction of the cystic fibrosis defect by retro virus-mediated gene transfer renders the resulting CFPAC-PLJ-CFTR cells insensitive to CPX. We now report that CPX also activates chloride efflux from the CF tracheal epithelial cell line 1B3-1 bearing a ΔF508 allele, but not if the 1B3-1 cells have been repaired by transfection of the wild type CFTR gene. Similar results were obtained with recombinant NIH 3T3 cells, in which CPX activates <sup>36</sup>[Cl-] efflux from cells expressing the CFTR, ΔF508 gene product, but not from 3T3 cells expressing the wild type CFTR. In all three cell types expressing CFTR, ΔF508, CPX was found to activate <sup>36</sup>[Cl-] efflux in a dose dependent manner over the concentration range of 1-30 nM, and then gradually lose potency at higher CPX concentrations. Six CPX analogues, A<sub>1</sub>-receptor antagonists of affinity similar to that of CPX, were found to be much less effective than CPX at activating <sup>36</sup>[Cl-] efflux from CFPAC-1 cells. These included 2-thio-CPX, CPT, 3,4-dehydro-CPX, 3-F-CPX, 3-1-CPX, and KW-3902. We conclude from these studies that CPX can activate chloride efflux from CF epithelial cells in

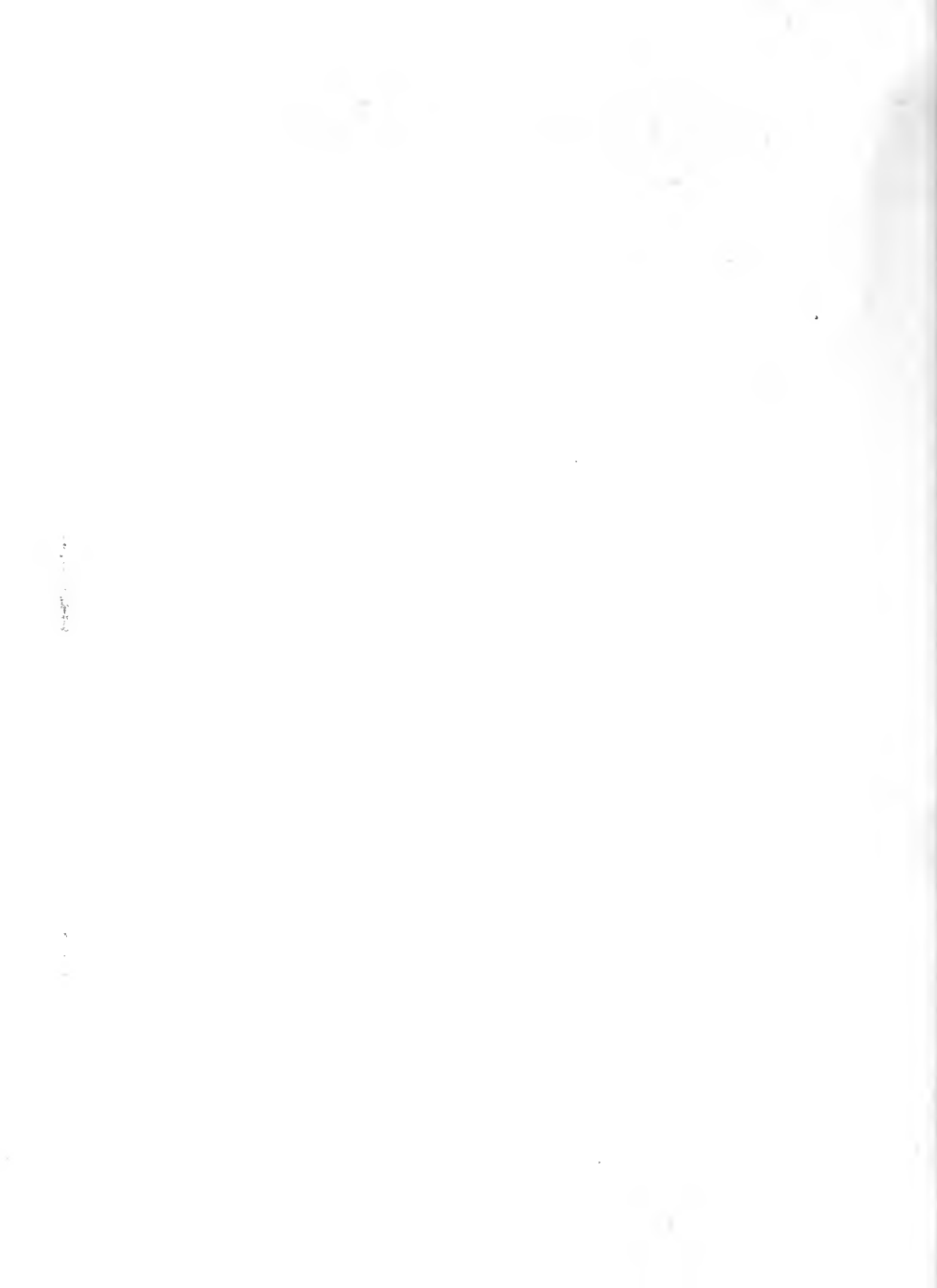




which the CFTR ( $\Delta F508$ ) genotype is present, and the wild type CFTR gene is absent. The presence of excess wild type CFTR in repaired CFPAC-1 and 3T3 (CFTR) cells was verified by Western blot analysis. In addition, the nature of the gene transfer vehicle does not seem to be important for the loss of sensitivity to CPX. The fact that this relationship can also be demonstrated with mouse 3T3 cells indicates that the CPX effect is not exclusively dependent upon a human epithelial cell substrate. Finally, the comparative data obtained with a variety of selective  $A_1$ -antagonists lead us to question the hypothesis that the CPX effects on CF cells occur via interactions with a classical  $A_1$ -receptor. Alternative possibilities include either direct action of CPX upon the  $\Delta F508$  mutant of CFTR, or action on a yet-to-be characterized purine binding site common to both human and mouse cells.

B. Hypotonicity Activated Chloride Conductance in Cystic Fibrosis (CF) and Non CF Pancreatic Cell Lines Studied by  $^{36}\text{Cl}$  Efflux and Patch Clamp

The CF locus encodes the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), which is thought to be an apical  $\text{Cl}^-$  channel regulated by phosphorylation and has also been suggested to have a regulatory function on other  $\text{Cl}^-$  channels. Activation of  $\text{Cl}^-$  and  $\text{K}^+$  conductances are involved in the regulatory volume response after cell swelling; this response seems to be conserved in CF. However, the cellular mechanism and the osmolarity-sensitive  $\text{Cl}^-$  channels have not been identified. Thus a role for CFTR in this process can not be excluded. To study the osmolarity-sensitive  $\text{Cl}^-$  pathways,  $^{36}\text{Cl}$  efflux and patch clamp experiments were done in PANC-1 (Normal) and CFPAC (CF) cells. The  $^{36}\text{Cl}$  efflux was measured during 10 minutes, in a medium with 10  $\mu\text{M}$  bumetanide, and where  $\text{CO}_2$  and bicarbonate were omitted and  $\text{Cl}^-$  was replaced by gluconate. The patch clamp experiments were done using the whole cell configuration with a Krebs solution in the bath and a CsCl solution in the pipet. In both cell lines hypotonicity increased the  $^{36}\text{Cl}$  efflux rates in a magnitude proportional to the reduction in osmolarity (range 18% to 50%). This stimulation was evident since the first 2 to 3 minutes of exposure to the hypotonic medium. In the electrophysiological experiments the response of the CF and non-CF cells was similar. A 20% reduction in the extracellular osmolarity induced rapid cell swelling and produced a stimulation of the currents with a delay of 5 to 10 min. Both the swelling and the currents increased progressively during all the hyposmotic challenge (no regulatory volume decrease was observed). The whole cell currents showed inward rectification and presented a slow inactivation pattern at depolarized voltages. The reversal potential was close to the calculated equilibrium potential for chloride. Replacing extracellular chloride



by gluconate, induced a shift in the reversal potential of about +40 mV and produced a marked reduction in the outward currents. After returning to the isosmotic medium the cell recovered the normal size but the currents slowly returned to the control values after 20 to 30 min. Both experimental techniques were coincident in showing that the hyposmolarity regulated chloride pathways are not altered in CF. The identification of the factors that cause the delayed activation of whole cell  $\text{Cl}^-$  currents, may be useful in explaining the mechanism that mediates the volume regulatory response in these cells.

## V. ALZHEIMER'S DISEASE AMYLOID ION CHANNELS

### A. Alzheimer Disease Amyloid $\beta$ Protein Forms Calcium Channels In Bilayer Membranes

Amyloid  $\beta$  protein ( $\text{A}\beta$ P) is the 40- to 42- residue polypeptide implicated in the pathogenesis of Alzheimer disease. We have incorporated this peptide into phosphatidylserine liposomes and then fused the liposomes with a planar bilayer. When incorporated into bilayers the  $\text{A}\beta$ P forms channels, which generate linear current voltage relationships in symmetrical solutions. A permeability ratio,  $P_{\text{K}}/P_{\text{Cl}}$ , of 11 for the open  $\text{A}\beta$ P channel was estimated from the reversal potential of the channel current in asymmetrical KCl solutions. The permeability sequence for different cations, estimated from the reversal potential of the  $\text{A}\beta$ P-channel current for each system of asymmetrical solutions, is  $P_{\text{Cs}} > P_{\text{Li}} > P_{\text{Ca}} > P_{\text{K}} > P_{\text{Na}}$ .  $\text{A}\beta$ P-channel current (either  $\text{Cs}^{2+}$  or  $\text{Ca}^{2+}$  as charge carriers) is blocked reversibly by tromethamine millimolar range) and irreversibly by  $\text{Al}^{3+}$  (micromolar range). The inhibition of the  $\text{A}\beta$ P channel current by these two substances depends on transmembrane potential, suggesting that the mechanism of blockade involves direct interaction between tromethamine (or  $\text{Al}^{3+}$ ) and sites within the  $\text{A}\beta$ P channel. Hitherto,  $\text{A}\beta$ P has been presumed to be neurotoxic. On the basis of the present data we suggest that the channel activity of the polypeptide may be responsible for some or all of its neurotoxic effects. We further propose that a useful strategy for drug discovery for treatment of Alzheimer disease may include screening compounds for their ability to block or otherwise modify  $\text{A}\beta$ P channels.

### B. Maxi-Conductances Observed For Alzheimer's Disease Amyloid $\beta$ -Protein Channels in Artificial Lipid Membranes

We have recently shown that the Alzheimer's Disease amyloid  $\beta$ -protein (A  $\beta$ , 140) forms cation channels in artificial lipid bilayers (P.N.A.S.(US) 90:567-571, 1993). Both



gating and conductance of these channels vary, depending on the cation carrying the current. We describe here that the A $\delta$ P channels can also exhibit large conductances in symmetrical KCl solutions with magnitudes as large as 5 nS. Channels of this size, such as complement, are usually cytotoxic. The number of preferred stable conductance levels appears to be voltage dependent, although we occasionally noted high frequency fluctuations between two high conductance states. We speculate that if such channels were formed in a cell membrane, the ensuing ion fluxes down their electrochemical potential gradients would saturate all known intracellular regulatory mechanisms.



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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 21019 11

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Mechanism of Hormone and Transmitter Secretion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Harvey B. Pollard, Chief, ICH, NIDDK, Others: G. Lee, H.D., Res. Chemist; E. Rojas, H.D., VS; I. Atwater, H.D., R.B.; M. Levine, M.D., Sen. Inv., Med. Off.; A. Burns, H.D., RB; M. Srivastava, H.D., SSF; C. McEntchen, H.D., res. Hys.; G. Kuipers, H.D., VA; M. Kukuljan, M.D., WF; G. Coping, RM Tech.; P. Washko, D.S., SV; D. Doron, M.D., SV; M. Adeyemo, H.D., SV; R. Vargas, D.S., FDA; K. Dharwal, H.D., SSF; R. Welch, H.D., IRTA; Y-H Wang, M.D., VF; Z. Zhang-Kock, H.D., SF; H. Cachu, B.S., Biologist; C. Gay-Broder, BS, Biologist; C. Roschero, H.D., VS; N. Arispe, H.D., VA; Y. raviv, H.D., VA; E. Olivares, M.D., VA; L. Vergara, M.D., VS; S. Pope, M.S. Microbio.; O. Eidelman, H.D., SV; R. Vinet, RD SV; N. Merizhinskaya, H.D., VA; P. Raynal, H.D., SV, E. Cohen, H.D., SV; A. Cardenas, H.D., SV

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Cell Biology and Genetics

SECTION

Section on Cell Biology and Biochemistry

INSTITUTE AND LOCATION

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TOTAL STAFF YEARS:

9.0

PROFESSIONAL:

8.0

OTHER:

1.0

CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Our work continues to focus on the processes leading to fusion between granule and plasma membranes during exocytotic secretion from cells, including chromaffin cells, beta cells from Islets of Langerhans, nerve terminals, and mucin secreting cells from tissues affected by cystic fibrosis. The contact and fusion processes during secretion may be mediated by the calcium binding protein synexin (annexin VII). We have learned that Annexin VII is immunolocalized to formed elements in the cytoplasm of chromaffin cells, including the chromaffin granule membranes. Annexins, I, II and V can also be detected in these and other cells, but their distribution if different from that of synexin. Potassium channels have also been shown to occur on chromaffin granule membranes, although they are not sensitive to calcium. The role of these channels may be to rearrange [K] in recycling granules following exocytosis. In pituitary gonadotrophs the potassium channels controlling the membrane potential are apamine sensitive, and regulate oscillations in membrane potential and internal calcium concentration. The mechanism of oscillation of calcium within these cells depends upon IP<sub>3</sub> metabolism. In islets of Langerhans, glucose has a complex effect on intracellular calcium and secretion. The steady state effect of glucose is to induce rhythmic oscillations in intracellular calcium and electrical potential, but the earliest action of glucose is to lower the calcium ion concentration. A transgenic mouse with yeast hexokinase in the insulin secretion, which is lacking in neonatal islets, but is induced by exposure of the islets of prolactin. We have also developed a potential drug for cystic fibrosis based on our observation that the Al-receptor antagonist, 8-cyclopentyl-1,3-dipropyl xanthine activates chloride efflux from human epithelial and mouse fibroblast cell lines bearing the CFTR (delta F508) mutation but not the wild type CFTR. We have also learned that the Alzheimer's disease amyloid forms ion channels in bilayer membranes, and that this activity may be the basis of amyloid neurotoxicity in this disease.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-22001-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Vitamin C: Biochemistry, Molecular Biology and Human Requirements

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Mark A. Levine	Senior Investigator	LCBG: NIDDK
Others: Kuldeep Dhariwal	Senior Staff Fellow	LCBG: NIDDK
Philip Washko	Staff Fellow	LCBG: NIDDK
Richard Welch	IRTA	LCBG: NIDDK
Yaohui Wang	Visiting Fellow	LCBG: NIDDK

COOPERATING UNITS (if any)

L. Cantilena (USUHS, Bethesda), C. Cantilena (Transfusion Medicine, CC, NIH) D. P. Begsten (Univ. Uppsala, Sweden), L. Helman (PB, NCI, NIH) and M. Vasquez (PS, NCI, NIH), K. Kirk (LC, NIDDK, NIH)

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TOTAL STAFF YEARS:

4.5

PROFESSIONAL:

4.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Optimal amounts of ascorbic acid (vitamin C) for maintaining human health are unknown. As a unique means to address this problem we developed the concept of in situ kinetics, in which the principles of reaction kinetics are applied to vitamin C dependent reactions in situ. Studies of catecholamine biosynthesis are the first proof that kinetics in situ can be characterized. The experiments also provide new evidence that reaction mechanisms and kinetics are different for reactions in situ compared to isolated reactions, and indicate that it is essential to study kinetics in situ to determine vitamin requirements. We previously discovered that ascorbic acid is accumulated by human neutrophils in mM concentration. We recently found that the physiologic structural analog glucose exquisitely regulates ascorbic acid transport by two distinct mechanisms. We also discovered that ascorbate accumulation in neutrophils can be upregulated 10 fold. The mechanism is by extracellular oxidation of ascorbate to dehydroascorbic acid, preferential transmembrane translocation of dehydroascorbic acid, and immediate intracellular reduction to ascorbate. These experiments are the first demonstration that ascorbate recycling occurs physiologically. To learn vitamin C function in human lymphocytes, experiments using differential hybridization and subtraction cloning are underway. Ascorbic acid accumulation in human fibroblasts was characterized as a prelude to understanding how different concentrations of the vitamin regulate proline hydroxylation. Clinical goals are to learn how much vitamin C is found in humans as a function of ingestion, so that ascorbic acid dependent reactions can occur. An IRB approved inpatient clinical trial has begun to learn for the first time how ascorbic acid ingestion regulates plasma and tissue concentrations in healthy humans. Five subjects who were inpatients for 4-6 months have completed the study and two additional subjects are inpatients. These data will provide new information and will permit human ascorbate requirements to be determined using in situ kinetics.





ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

I. POLYAMINES

We have now shown that spermidine is absolutely required for protection against oxidative damage from superoxide formation. When polyamine deprived cells are grown in 95% oxygen there is a dramatic loss in cell viability unless spermidine is added. This loss in cell viability is not seen if the strain also contains an expression plasmid for superoxide dismutase, indicating the involvement of superoxide in the sensitivity of the polyamine-deficient cells to oxygen. The requirement for spermidine is not satisfied by endogenous or exogenous putrescine.

The protective effect of spermidine against oxygen toxicity is not the only physiological function of spermidine, since the *spe2* deletion mutant still requires spermidine for growth, even if the cells are grown in air or anaerobically.

. . . . . Drs. D. Balasundaram, C. W. Tabor and H. Tabor

We have previously shown the importance of polyamines *in vivo* for protein synthesis in *E. coli* by showing a requirement for polyamines for the readthrough of an amber codon in bacteriophages T4 and T7.

We now show the involvement of spermidine in another type of translational control; namely, ribosomal frameshifting in *Saccharomyces cerevisiae*. These studies were carried out with Drs. Wickner and Dinman, using the system they have developed and extensively used for studying frameshifting in yeast. Using their model plasmids containing the L-A or Tyl frameshift signals, we found that the efficiency of +1 (but not -1) frameshifting is dramatically increased in yeast cells upon depletion of spermidine and spermine.

In related studies with Dinman and Wickner we showed that the elevated +1 frameshifting observed during polyamine depletion leads to a marked loss of Tyl transposition. Relatively high concentrations of spermidine are required to maintain a 'wild type' transposition frequency. Presumably these results reflect the need for the correct ratio of the Tyl gene products (TyA and TyB), i.e. as has been shown by Dinman and Wickner for the gag and pol proteins in the L-A system in yeast.

On the basis of these data we postulate that spermidine is very important for the functional integrity of the ribosomal protein synthesizing complex either because of its effect on the structure of the complex or because polyamines are important for the synthesis of the aminoacyl-tRNA involved in this complex.

These studies are of interest, not only because of their demonstration of a new and unique physiological function for spermidine, but also because of the implication for possible antiviral therapy, since retroviruses are known to require ribosomal frameshifting for the proper synthesis of the gag-pol proteins.

. . . . . Drs. J. Dinman, R. B. Wickner, D. Balasundaram, C. W. Tabor and H. Tabor



The ability of *spe2Δ* cells to utilize glycerol as an energy source is lost when these cells are grown in amine-deficient media in air, indicating the loss of functional mitochondria. This loss of functional mitochondria is permanent and is not reversed when the cells are placed in media containing spermidine.

No loss of mitochondrial function is seen if the incubation is carried out anaerobically.

. . . . . Drs. D. Balasundaram, H. Tabor and C. W. Tabor

We have isolated a number of suppressor mutants of the *spe2Δ* strain that can grow, albeit very slowly, in the absence of polyamines. These strains do not lose their mitochondria during the polyamine deprivation, nor do they show the toxicity of oxygen described above. Preliminary mapping of this bypass mutation indicates that it is on chromosome XV, very close to the *SPE2* gene.

. . . . . Drs. D. Balasundaram, H. Tabor, and C. W. Tabor

We had previously prepared a yeast strain that cannot synthesize any putrescine, spermidine, or spermine because of deletion-insertion mutations in the ornithine decarboxylase gene (*spe1Δ*) and the S-adenosylmethionine gene (*spe2Δ*). We prepared an isogenic strain that contains a plasmid (pSPE2.3) that overexpresses the wild-type gene for S-adenosylmethionine decarboxylase. Although neither strain could synthesize any of the polyamines since no putrescine can be synthesized due to the *spe1* deletion, only the latter mutant could synthesize decarboxylated S-adenosylmethionine. Surprisingly, the latter mutant showed the effects of polyamine-deficiency much earlier than the isogenic strain without the pSPE2.3 plasmid. With an HPLC method that we developed, we found that only the culture that contained the SPE2.3 plasmid accumulated decarboxylated adenosylmethionine suggesting that decarboxylated adenosylmethionine is toxic in polyamine deficient yeast.

These observations may have practical significance since numerous studies have been carried out on ornithine decarboxylase inhibitors as anticancer agents. Our results indicate that the efficacy of such inhibitors may be influenced by the activity of S-adenosylmethionine decarboxylase.

. . . . . Drs. D. Balasundaram, H. Tabor and C. W. Tabor

Previous work on the isolation of this enzyme from yeast was complicated by the presence of proteases in yeast. Therefore the enzyme has now being prepared from a yeast strain lacking the major proteases. Also studies are being carried out on the regulation of ornithine decarboxylase and the relationship of the *SPE1*, *SPE4* and *SPE40* genes.

. . . . . Drs. H. Tabor and C. W. Tabor

Studies have been carried out on a strain containing a mutation in *SPE3*, the gene for spermidine synthase. Preliminary results have been done on the cloning and expression of this gene from a yeast gene bank.

. . . . . Drs. C. W. Tabor, H. Tabor and N. Hamasaki



## II. YEAST VIROLOGY

*The SKI antiviral system.* We have described a system of six chromosomal genes (*SKI* for superkiller) that negatively control the copy number of three completely unrelated cytoplasmic RNA replicons all lacking 5' cap and 3' polyA: L-A dsRNA (and its satellites M and X dsRNAs), L-BC dsRNA, and 20S RNA (a ssRNA replicon). The same system does not affect retrotransposition by Ty1 which is transcribed as mRNA with 5' cap and 3' polyA. We now find that the *SKI2* gene encodes a 145 kDa protein with helicase motifs and a glycine-arginine rich region typical of nucleolar proteins. We find that *SKI2* represses expression from viral mRNA lacking 5' caps and 3' polyA, but not the same information expressed from cDNA clones. In support of the hypothesis that the *SKI* system recognizes the absence of 5' caps or 3' polyA structure, we find that expression of -galactosidase from an rDNA-LacZ fusion construct, that should be expressed by uncapped mRNA lacking 3' polyA, is repressed by the *SKI* system. The *SKI* genes are only necessary for repression of viral propagation. We suggest that cells defend themselves against a broad range of viral infections by blocking translation of RNA lacking 5' cap and/or 3' polyA.

. . . . . Drs. W. R. Widner and R. B. Wickner

*Mammalian - Yeast chimeric RNA dependent RNA polymerase can support a dsRNA replicon in yeast.* We have substituted the RNA polymerase consensus domain of the L-A dsRNA virus with that of the mammalian Sindbis (+) ssRNA virus and found that the resulting chimeric enzyme is capable of propagating the M<sub>1</sub> satellite dsRNA at 20° C. These findings support the idea that the L-A RNA-dependent RNA polymerase has more than a distant phylogenetic relationship to those of the hundreds of (+) ssRNA viruses and dsRNA viruses that share consensus sequence patterns with L-A, many of which are important human pathogens.

. . . . . Drs. J. C. Ribas and R. B. Wickner

*Chromosomal genes affecting -1 ribosomal frameshifting.* Like retroviruses (including HIV), the L-A dsRNA virus uses a -1 ribosomal frameshift to make its Gag-Pol fusion protein. We have shown that the efficiency of this event is critical for viral propagation. We have now isolated mutants in eight chromosomal genes resulting in increased efficiencies of -1 ribosomal frameshifting and consequently loss of M<sub>1</sub> dsRNA. That cell growth is less sensitive to increases in ribosomal frameshifting than viral propagation suggests a possible approach to antiretroviral drugs.

. . . . . Drs. J. D. Dinman and R. B. Wickner

*Exclusion of L-A virus by an L-A cDNA clone.* Expression of a full-length cDNA clone of the L-A dsRNA virus causes virus loss, independent of the presence of the packaging site or of cis sites for replication and transcription on the cDNA clone, but completely dependent on expression of functional recombinant gag and gag-pol fusion protein. We suggest that exclusion is due to competition by proteins expressed from the plasmid for a possibly limiting cellular factor.

. . . . . Drs. R. Valle and R. B. Wickner



*Evidence for functionally distinct classes of ribosomes.* We find that the MAK7 gene, necessary for M<sub>1</sub> propagation, is identical to RPL4A, a gene for ribosomal protein L4. A duplicate gene exists, RPL4B, which differs in 7 amino acid residues of 256 from RPL4A, and is expressed at a higher level than RPL4A. Deletion of RPL4B does not affect propagation of M<sub>1</sub>, nor does overexpression of RPL4B suppress deletion of RPL4A, indicating that the two L4 proteins are functionally distinct. Since each ribosome has one copy of each ribosomal protein, this shows that there are functionally distinct classes of ribosomes.

. . . . . Drs. Y. Otake and R. B. Wickner

*Structure of the L-A virus.* Cryo-electron microscopy and 3D image reconstruction has been used to show that the L-A virus has icosahedral symmetry with a T=1 structure. The assymetric unit is a dimer of Gag molecules.

. . . . . Drs. J. R. Caston (LSB, NIAMS), B. L. Trus (LSB, NIAMS), N. Cheng (LSB, NIAMS), A. C. Steven (LSB, NIAMS), R. B. Wickner, G. Wang (Purdue U.), T. J. Smith (Purdue U.), R. H. Cheng (Purdue U.), and T. S. Baker (Purdue U.)

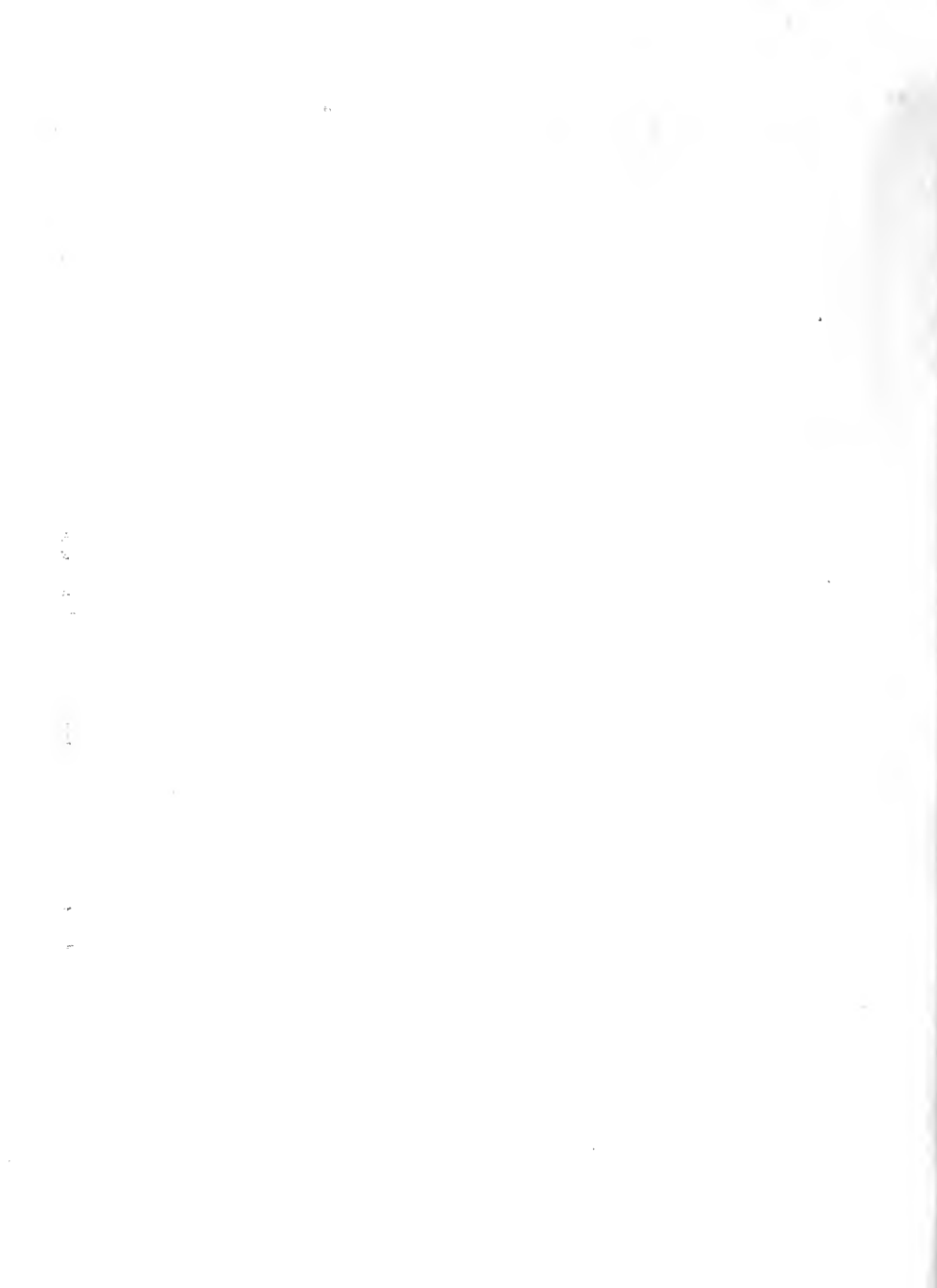
*Covalent attachment of 5' caps from cellular mRNAs to Gag protein.* L-A virus particles catalyze the *in vitro* covalent attachment of the 5' cap structure of any RNA to the Gag protein. We find that the cap structure becomes covalently attached to histidine 154 of Gag. Mutation of His154 to Arg, Asn or Ser results in inability of the L-A cDNA clone to support the propagation of the M<sub>1</sub> satellite RNA.

. . . . . Drs. A. Blanc (McGill U.), N. Sonenberg (McGill U.) and R. B. Wickner

### III. GENOMIC STRUCTURE AND FUNCTION

L1 DNA (long interspersed repeated DNA, LINE 1 DNA) is a self replicating parasite of mammalian genomes and accounts for at least 10-20% of mammalian DNA. L1 elements contain a regulatory sequence at the left end, two highly conserved genes (one of which encodes a reverse transcriptase), and a guanine-rich polypurine:polypyrimidine sequence near the right end. Transposition of L1 elements is a frequent cause of polymorphism in mammals including humans where it has caused genetic defects. We have been studying the L1 family of rats and describe below our recent findings.

*Abnormal DNA structures: Triplexes, Z-DNA and cruciforms.* Sequences with the potential to form abnormal DNA structures have been found in the promoters and coding sequences of a number of different genes in prokaryotes and eukaryotes. We have shown that the sequences at the right end of mammalian L1 elements and in their target sites can adopt a variety of abnormal DNA structures. The nature of these structures together with their requirement for supercoil energy suggests a number of possible roles for these sequences *in vivo*. We had previously shown that these structures affect chromatin assembly, are sensitive to strand-cleavage by endogenous enzymes, act as barriers to DNA replication *in*





*vitro* and compete effectively with other supercoil dependent structures for supercoil energy. We have now shown that such sequences can indeed inhibit DNA replication, stimulate illegitimate recombination and inhibit transcription *in vivo*.

We have constructed a new, very sensitive assay system to study factors that affect deletion, insertion and triplet-expansion mutations. Abnormal DNA structures are known to be very sensitive to certain carcinogens and heavy metals *in vitro*. Work is now underway to study the sensitivity of these sequences to these compounds *in vivo*.

The ability of these abnormal DNA structures to compete for supercoil energy may provide a novel means of regulating genes driven by supercoil dependent promoters. To test this hypothesis, we are using plasmids that contain both a sequence with abnormal DNA structure-forming potential and a supercoil-dependent promoter directing expression of a reporter gene. We have identified a number of promoters that are potential candidates for this mode of regulation. Molecular dissection of these promoters will help us understand the role of abnormal DNA structures on gene expression.

. . . . Drs. K. Usdin and R. Howell

*The L1 regulatory region.* We previously showed that the left-most ~600 bp of rat L1 DNA resembles a transcriptional regulatory sequence in that it is a CpG island and can stimulate the activity of the chloramphenicol acetyl transferase (CAT) gene fused to it. To understand how the regulatory sequence works, we analyzed both its structure and the fusion transcripts synthesized *in vivo*. Although the L1 regulatory sequence does not contain binding sites for any of the known transcriptional activation factors, two regions of it can form specific complexes with partially purified nuclear factors present in rat cells. One of the protein binding regions, which is conserved between different rat L1 subfamilies, also binds nuclear factors present in primate cells. Although deletion of this region completely eliminated the protein binding activity of the L1 regulatory region, its stimulation of CAT gene activity was undiminished. Also, deletion of the other protein binding region did not effect the stimulation of CAT gene activity. One explanation for the disjunction between the nuclear protein binding activity and the CAT gene stimulatory activity is that the L1 regulatory sequence is not a typical transcriptional activation sequence. Our transcriptional analysis verified this supposition: First, transcripts are initiated about 800 bp 5' of the beginning of the L1 regulatory sequence in a cryptic promoter site within the fusion vector. This site is also active in the control fusion vector that lacks the L1 regulatory sequence. Second, there is no difference between the amount of CAT gene transcripts in cells transfected with the control fusion vector and with a fusion vector containing the regulatory region of two different L1 subfamilies, which stimulate CAT activity anywhere from 6 to 20 times, depending on the sequence used. Therefore, this stimulation is entirely due to a post transcriptional effect of the L1 regulatory sequence. This effect is both position and orientation dependent, indicating that the presence of the "sense" L1 sequence within the transcript is required. We have constructed deletion derivatives of the L1 regulatory sequence that completely abolish its stimulatory effect, but have no effect on the amount of transcripts. We are now studying the mechanism of this post transcriptional stimulatory effect



. . . . . Drs. B. Hayward, M. Zavanelli, and A. V. Furano

*Evolution of L1 elements.* The use of L1 amplification events as a phylogenetic tool. We previously showed that the L1 population of modern rodents consists of both recently evolved L1 elements and the relics of their ancestors. Some of these had been highly amplified and therefore provide an easily detected and seemingly unequivocal "fossil" record of the phylogenetic history of these animals. The following two examples establish that L1 fossils are indeed an excellent source of phylogenetic information:

The ancestral L1 family, Lx, was amplified about 12 million years ago during the radiation of the Murinae and we found the relics of this amplification in 22 of 38 rodent species examined. Twenty of the Lx-positive species had been previously classified as Murinae and include the oldest known genus of the Murinae, *Micromys minutus*. The 2 remaining Lx-positive species are African Vlei rats. Although traditionally classified as a separate Muridae subfamily equal in rank to the Murinae, our results indicate that the Otomyinae genera actually comprise an African clade of the Murinae. Lx relics were absent from *Acomys cahirinus*, *Uranomys ruddi*, and *Lophuromys flavopunctatus*. Although these species were traditionally classified as Murinae on morphological criteria, our results indicate that this was also incorrect. The reclassifications prompted by our data are also supported by single copy DNA hybridization data and a re-examination of the morphological data and fossil evidence. Therefore, the Lx amplification event is a defining characteristic, or synapomorphy, of the true Murinae.

Amplification of modern L1 subfamilies can serve as a synapomorphy for more recently diverged species. The Mlvi2r subfamily of the modern rat L1 family has been amplified in just 2 of 7 *Rattus* species examined: *Rattus norvegicus* and in 3 individuals identified as *Rattus rattus* (Moluccarius), thereby defining a new clade within the genus *Rattus*. Our phylogenetic analysis of the seven *Rattus* species using mitochondrial DNA sequence data corroborated the existence of this new Mlvi2r-containing clade.

. . . . . Drs. A. V. Furano, B. E. Hayward and K. Usdin in collaboration with François M. Catzeflis

*Coordinate evolution of two structures in a novel modern L1 subfamily.* The repeated and episodic amplification of L1 elements indicates that novel, amplification-proficient L1 subfamilies have repeatedly emerged during mammalian evolution. The Mlvi2r family (see section A) is just such a subfamily. We isolated 4 full length members from this newly evolved family and compared them with members of its immediate predecessor, the canonical modern L1 family in *Rattus* that we had previously characterized. The major differences between the two subfamilies were confined to just two locations: One was in ORF I, in which 35 bp of the coding sequence had been changed by insertion and rearrangement events. The second was in the regulatory sequence and involved clustered base changes in several regions, two of which overlapped with the regions that we had identified by deletion analysis to be essential for the stimulatory effect of the regulatory region on CAT gene activity (see section II). The coordinate evolution of the regulatory sequence and ORF I strongly suggests that the ORF I protein is



involved in the stimulatory effect of the regulatory region. We are now testing whether this supposition is correct.

. . . . . Drs. B. Hayward, M. Zavanelli, K. Usdin, and A. V. Furano

#### IV. MEMBRANE STUDIES OF *ESCHERICHIA COLI*

**Aldoheptose Biosynthesis.** Gram-negative bacteria (pathogens and nonpathogens) have a unique outer membrane and are more refractory to therapeutic attack than their gram-positive counterparts, save mycobacteria. Lipopolysaccharide (LPS) forms all of the lipid of the exterior leaflet of this unique outer membrane. Mutants defective in the LPS inner core aldoheptose (i.e., L-glycero-D-mannoheptose) are hypersensitive to a number of antibiotics. The molecular genetics and biology of the *E. coli* K-12 *rfaD* gene have been reported by this laboratory. The purified *rfaD* gene product has been characterized both kinetically and physically. Crystals of *rfaD* gene product (ADP-L-glycero-D-mannoheptose 6-epimerase) have been obtained. The ubiquity of the *rfaD* gene among enteric and nonenteric gram-negatives is under study. For example, we have demonstrated structural and functional homology of the *rfaD* gene of nonenteric pathogen *Pseudomonas aeruginosa*. The molecular genetics and biology of a second mutation, recently designated *rfaC* (formerly *rfa-2*), clearly defines the *rfaC* gene and its product (heptosyl transferase 1). The opening frames for both the *rfaF* and *rfaL* genes and their chromosomal relationship to *rfaC* and *rfaD* genes have been determined. An affinity chromatography based purification protocol for phosphoheptose isomerase has been developed. New mini-Tn10dCAM insertion *rfa* novobiocin hypersensitive or barrier mutants have been isolated and are now subjects of investigation.

. . . . . Drs. W. G. Coleman, Jr., L. Chen, L. Ding and K. Crawford

#### V. ENZYME STRUCTURE AND FUNCTION

We are using the tryptophan synthase multienzyme  $\alpha\beta_2$  complex as a model system for investigating how protein-protein interaction and protein-ligand interaction affect enzyme structure and function. Tryptophan synthase is also an excellent system for investigating enzymatic reaction mechanisms and metabolite channeling. Our previous X-ray crystallographic studies of the wild type  $\alpha\beta_2$  complex from *Salmonella typhimurium* reveal that the active sites of the  $\alpha$  and  $\beta$  subunits are 25 Å apart and are connected by a tunnel. Thus reciprocal communication between the active sites of the heterologous subunits occurs over 25 Å and must involve protein conformational changes. We are using the crystal structure as a framework for selecting enzyme residues for site-directed mutagenesis. During the past year we have used several experimental techniques to investigate intersubunit communication and putative conformational changes in the wild type and mutant enzymes.

**Three-Dimensional Structures of Wild Type and Mutant Forms of the Tryptophan Synthase.** The structures of the wild type  $\alpha\beta_2$  complex with different ligands have been refined to about 2 Å resolution. Stable enzyme-substrates intermediates of a mutant  $\alpha\beta_2$  complex (K87T) with L-serine and L-tryptophan have been crystallized and analyzed to high



resolution. The results reveal the location of the substrate binding site in the  $\beta$  subunit and identify important ligand-induced conformational changes. Studies of additional mutants are underway. Diffraction studies of crystals of the wild type  $\beta$  subunit have been initiated. A comparison of the structures of the  $\beta$  subunit and  $\alpha_2\beta_2$  complex would elucidate the conformational changes that occur upon association of the  $\beta$  subunit with the  $\alpha$  subunit.

. . . . . Drs. E. W. Miles and S. A. Ahmed with Drs. K. Parris and D. R. Davies (LMB, NIDDK) and C. C. Hyde (LSBR, NIAMS).

*Steady State and Rapid Kinetic Studies of Intersubunit Communication and Channeling.* Mutations of Glu-49, Asp-60, Gly-51, and Arg-179 in the  $\alpha$  subunit alter the kinetics of reactions at the active site of the  $\beta$  subunit, alter the effects of  $\alpha$  specific ligands on kinetics, and alter the kinetics of metabolite channeling. The results provide evidence that these mutations interfere with a conformational change that results in the conversion of the wild type  $\alpha$  subunit from an open form to a closed form. A flexible loop in the  $\alpha$  subunit (loop 6) plays an important role in this conformational transition and in intersubunit communication.

. . . . . Dr. E. W. Miles with Dr. C. C. Hyde (LSBR, NIAMS) and Drs. P. S. Brzovic' and M. F. Dunn (University of California Riverside)

*Fluorescence Studies of Intersubunit Communication.* We have exploited the fluorescence properties of Nile Red to probe the effects of ligands and of limited proteolysis on the conformations of the  $\alpha$  and  $\beta$  subunits in the  $\alpha_2\beta_2$  complex. The fluorescence emission intensity of Nile Red at a hydrophobic  $\beta$  subunit site in the  $\alpha_2\beta_2$  complex is dramatically decreased by addition of an  $\alpha$  subunit ligand. Tryptic cleavage of  $\alpha$  subunit loop 6 blocks the effects of allosteric ligands on the conformational properties of the  $\beta$  subunit. We are using other intrinsic and extrinsic fluorophores to probe conformational transitions in tryptophan synthase.

. . . . . Drs. E. W. Miles, S. B. Ruvinov, and U. Banik with Dr. D. L. Sackett

*Critical Role of  $\beta$  Subunit Lys-167 in Intersubunit Communication.* An engineered mutation in the contact region between the  $\alpha$  and  $\beta$  subunits remarkably alters the catalytic and spectroscopic properties of the  $\beta$  subunit in the  $\alpha_2\beta_2$  complex. Ligands that bind to the  $\alpha$  subunit largely reverse the effects of mutation. We propose that the ligands induce conformational changes in the  $\alpha$  subunit that are transmitted to the  $\beta$  subunit and result in repair of the mutational defect in the  $\beta$  subunit.

. . . . . Drs. E. W. Miles and X.-J. Yang

*Effects of Protein-Protein Interaction and of Protein-Ligand Interaction of the Thermal Stability of Tryptophan Synthase.* Differential scanning calorimetric studies show that the unfolding pathway of the  $\alpha_2\beta_2$  complex is more complex than that of the separated  $\alpha$  and  $\beta$  subunits. These results shed light on the effects of subunit interaction on structure and thermal stability. Studies of thermal inactivation show that ligands have striking effects on subunit interaction and on thermal stability.

. . . . . Drs. E. W. Miles and S. B. Ruvinov with Drs. A. Ginsburg and D. P. Remeta, LB, LHLBI





*Mechanistic Roles of Lysine-87 in the  $\beta$  Subunit.* We have used site-directed mutagenesis has been used to probe the functional roles of the lysine residue that forms a Schiff base with pyridoxal phosphate coenzyme of the  $\beta$  subunit. Spectroscopic and kinetic studies of a mutant form of the  $\beta$  subunit (K87T) provide evidence that Lys-87 serves critical roles in transamination, catalysis and product release. The K87T  $\alpha_2\beta_2$  complex forms a stable L-serine Schiff base that has been used for x-ray crystallography. Addition of ammonia converts the Schiff base of L-serine to the Schiff base of aminoacrylate by nonenzymatic "chemical rescue". Kinetic studies show that the Schiff base of aminoacrylate is the key intermediate which triggers a conformation change that activates the  $\alpha$  subunit.

. . . . . Drs. E. W. Miles, Z. Lu, U. Banik and P. McPhie

*Conformations of Tryptophan Synthase.* Studies of the inactivation of wild type and mutant forms of tryptophan synthase by  $\beta$ -chloro-L-alanine provide evidence that the wild type  $\beta$  subunit is converted from an "open" form to a "closed" form upon association with the  $\alpha$  subunit. Certain mutant forms of the  $\beta$  subunit do not undergo this conformational change upon association with the  $\alpha$  subunit. Moderate concentrations of solvents (ethanol or 2-mercaptoethanol) also prevent conversion of the wild type  $\alpha_2\beta_2$  complex to the closed form. Inactivation studies provide evidence that the  $\beta$  subunit exists in at least two conformers in solution.

. . . . . Drs. S. A. Ahmed and E. W. Miles

This laboratory is engaged in studies on protein structure and the mechanism of protein folding. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight= 39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of its first 44 amino acids, to produce an enzymatically active protein, pepsin. Pepsin is stable only at pH's below 6. Pepsin and pepsinogen are unfolded by exposure to high pH, temperature or concentrations of denaturants, such as urea. However, unfolded pepsinogen can refold to its normal structure, when returned to native conditions, whereas pepsin cannot. I am interested in the mechanism of this refolding reaction and how the difference in sequence influences the refolding of the two proteins. We have used techniques such as ultraviolet, circular dichroic and fluorescence spectroscopies, together with chemical modification and peptide chemistry, to characterize the structures of the native and unfolded species. We have used rapid kinetic techniques, such as stopped-flow and T-jump, to detect partly folded forms in the folding reaction; their structures have been partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.

. . . . . Dr. P. McPhie with Dr. W. Jakoby, LBM, Drs. J. Cheng and S. Adhya, LMB, NCI, and Dr. V. Ivanov, Engelhart Institute of Molecular Biology, Moscow

## VI. PHYSICAL BIOCHEMISTRY

The calcium-linked hetero-association of human complement subcomponents C1r and C1s has been quantitatively characterized as a function of calcium concentration via the technique of tracer sedimentation equilibrium.

. . . . . Drs. G. Rivas, K. Ingham and A. P. Minton



The role of macromolecular crowding in the regulation of cellular volume has been further explored.

. . . . . Dr. A. P. Minton

A new technique for the detection of associations in mixtures of several protein components, called sedimentation equilibrium - quantitative polyacrylamide gel electrophoresis (SE-QPAGE), has been developed and tested on mixtures of up to four protein components.

. . . . . Drs. S. Darawshe, G. Rivas, and A. P. Minton

Theoretical models have been proposed for the (i) effect of confining proteins to small pores of different shapes, and (ii) and the effect of weak nonspecific attractive interactions between surfaces and proteins upon various conformational and association equilibria.

. . . . . Dr. A. P. Minton

Cooperative binding systems are being studied taking into account site or subunit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems, particularly those involving the action of fibrinogen and the processes of protein-DNA interactions.

. . . . . Drs. H. Saroff and E. Mihalyi

## VII. ENDOCRINE BIOCHEMISTRY

Labeled  $\beta$ -tubulin from tubulin irradiated in the presence of labeled colchicine can be separated and purified from SDS preparative gels and analyzed by proteolysis. Both proteases yielded a labeled ~4kDa band containing two peptides. Sequence analysis revealed a peptide of residues 1-20 or 1-36 and 213-242 for chymotrypsin, and 1-19 or 1-46 and 214-241 for trypsin. To identify which peptide carried the label, limited hydrolysis of  $\beta$ -tubulin was carried out with trypsin; this yielded a labeled 16kDa N-terminal peptide and a 35kDa C-terminal peptide as identified by antibodies. Isolation of these peptides and extensive digestion with trypsin yielded two labeled peptides corresponding to residues 1-19 or 1-46 from the 16kDa N-terminal fragment, and residues 214-242 from the 35kDa C-terminal fragment. These results show that at least two domains in  $\beta$ -tubulin are specifically involved in colchicine binding and that the span of the colchicine molecule,  $\leq 11\text{\AA}$ , bridges these two domains in the native  $\beta$ -monomer.

. . . . . Drs. J. Wolff and S. Uppuluri

We have sought to define the changes in tubulin structure that are brought about by colchicine binding. A single new cleavage product is found when either enzyme is used to digest the tubulin-colchicine complex. The new fragments from both enzymes were nearly the same size ( $M_r = 43,000$ , compared to the undigested subunit  $M_r = 50,000$ ). Monoclonal antibodies with known epitopes revealed that both the tryptic and chymotryptic fragments contained the amino terminus but not the carboxyl terminus of  $\beta$ -tubulin. The masses of both fragments were determined by multiple gel



electrophoresis and by electrospray mass spectrometry. The cleavages occur after lys-392 for trypsin and phe-389 for chymotrypsin. Secondary structure analysis indicates that this region is alpha-helical, and the cleavages occur at the carboxyl end of this helix. The helix has strong amphipathic character. The carboxyl end of the amphipathic helix must be unfolded by colchicine binding to allow cleavage to occur. We suggest that this local unfolding of the protein may prevent proper formation of the inter-dimer contact essential for polymerization and explain colchicine's biological actions, including the known substoichiometric poisoning of microtubule assembly.

. . . . Dr. D. Sackett and J. Varma (Harvard University Medical School)



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 23,140-35 LBP

## PERIOD COVERED

October 1, 1992, through September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemistry of Sulfur-Containing Compounds

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: Simon Black, Ph.D.

Chemist (Research)

LBP NIDDK

Others: Dorothy Black

Special Volunteer

LBP NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Pharmacology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL STAFF YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

(a) Human subjects

(b) Human tissues

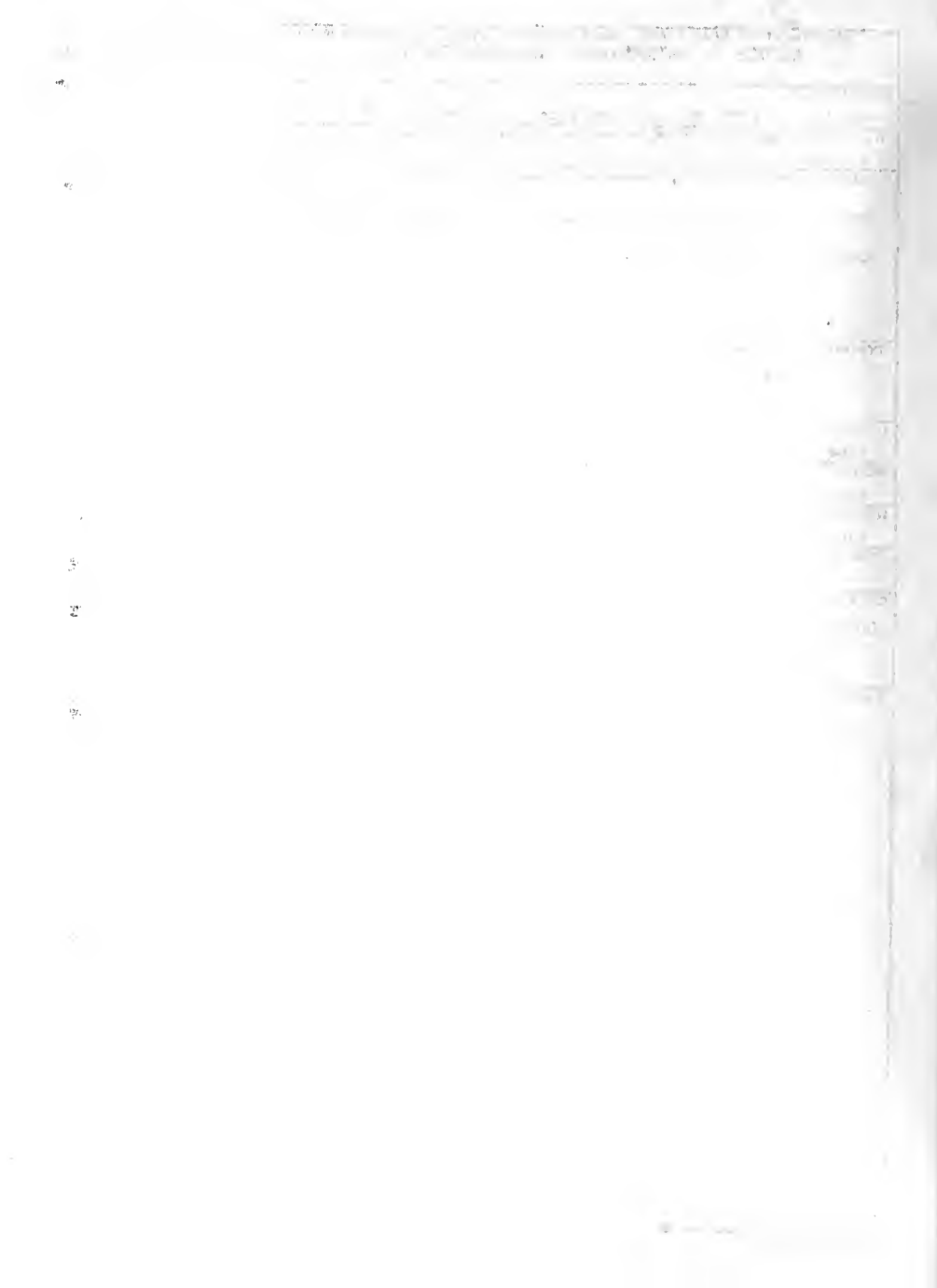
✓ (c) Neither

(a1) Minors

(a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 23,330-15 LBP
PERIOD COVERED October 1, 1992, through September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The permeability barriers of gram-negatives and mycobacteria to antibiotics and chemotherapeutics		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation) PI: William G. Coleman, Jr., Ph.D. Research Microbiologist LBP NIDDK Others: Lishi Chen, Ph.D. Visiting Associate LBP NIDDK Li Ding, Ph.D. Visiting Fellow LBP NIDDK Keith Crawford, Ph.D. IRTA Fellow LBP NIDDK		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Pharmacology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS 4.6	PROFESSIONAL: 3.8	OTHER: 0.8
CHECK APPROPRIATE BOX(ES) (a) Human subjects      (b) Human tissues <input checked="" type="checkbox"/> (c) Neither (a1) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p><b>Aldoheptose Biosynthesis.</b> Gram-negative bacteria (pathogens and nonpathogens) have a unique outer membrane and are more refractory to therapeutic attack than their gram-positive counterparts, save mycobacteria. Lipopolysaccharide (LPS) forms all of the lipid of the exterior leaflet of this unique outer membrane. Mutants defective in the LPS inner core aldoheptose (i.e., L-glycero-D-mannoheptose) are hypersensitive to a number of antibiotics. The molecular genetics and biology of the E. coli K-12 rfaD gene have been reported by this laboratory. The purified rfaD gene product has been characterized both kinetically and physically. Crystals of rfaD gene product (ADP-L-glycero-D-mannoheptose 6-epimerase) have been obtained. The ubiquity of the rfaD gene among enteric and nonenteric gram-negatives is under study. For example, we have demonstrated structural and functional homology of the rfaD gene of nonenteric pathogen <i>Pseudomonas aeruginosa</i>. The molecular genetics and biology of a second mutation, recently designated rfaC (formerly rfa-2), clearly defines the rfaC gene and its product (heptosyl transferase 1). The opening frames for both the rfaF and rfaI genes and their chromosomal relationship to rfaC and rfaD genes have been determined. An affinity chromatography based purification protocol for phosphoheptose isomerase has been developed.</p> <p>New mini-Tn10dCAM insertion rfa novobiocin hypersensitive or barrier mutants have been isolated and are now subjects of investigation.</p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 23,580-30 LBP
PERIOD COVERED October 1, 1992, through September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mammalian Transposons		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Anthony V. Furano, M.D. and Chief, Section on Genomic Structure and Function	Medical Officer (Research) LBP NIDDK
Others:	Karen Usdin, Ph.D. Bruce E. Hayward, Ph.D. Mary Zavanelli, Ph.D. Renée Howell, Ph.D. Kerry Woodford	Visiting Associate Visiting Associate IRTA IRTA Summer IRTA LBP NIDDK LBP NIDDK LBP NIDDK LBP NIDDK LBP NIDDK
COOPERATING UNITS (if any) Dr. Francois Catzeflis, Institute of Evolutionary Science, Montpellier, France; Dr. Michael Seidman, Otsuka Pharmaceuticals, Rockville, MD		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Genomic Structure and Function		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS	PROFESSIONAL:	OTHER:
5.5	4.8	0.7
CHECK APPROPRIATE BOX(ES) (a) Human subjects      (b) Human tissues <input checked="" type="checkbox"/> (c) Neither (a1) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  Members of the L1 transposon family (long interspersed repeat DNA or LINE family) of rats are 6.7 kb long, 5 kb of which is devoted to two protein encoding genes. A regulatory region is at the left end of the element, and a guanine-rich polypurine:polypyrimidine sequence is near the right end. The protein encoding sequences of mammalian L1 families are highly conserved, but the regulatory sequences are completely distinct. Therefore, these families have been independently amplified in various mammalian species. We discovered the existence of ancient L1 families and our studies of them and their modern counterparts showed that extensive amplification of L1 elements has been episodic and recurrent during mammalian evolution, and that L1 amplification events provide very robust phylogenetic information. We previously showed that the rat L1 element regulatory region strongly stimulates the activity of a gene fused to it which was the first evidence that L1 DNA is not just some non-functional "junk" DNA. Although the DNA of the regulatory region can form specific complexes with nuclear DNA-binding proteins, the L1 regulatory region is not a typical transcriptional activator sequence since it stimulates gene activity solely by a post transcriptional mechanism. We earlier showed that the L1 guanine-rich polypurine:polypyrimidine sequence destabilizes contiguous duplex DNA, adopts several non-B DNA structures <i>in vitro</i> , and that it competes with target site non-B structures for supercoil energy which <i>in vivo</i> might modulate the supercoil dependent properties of L1 elements and their target sites. We now find that the L1 polypurine:polypyrimidine sequence decreases the replication of plasmids in both bacteria and mammalian cells and alters the apparent activity of certain eukaryotic promoters <i>in vivo</i> . In parallel studies we have shown that homoguanine stretches enhance the mutation and recombination rate of adjacent DNA sequences <i>in vivo</i> .		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 23, 750-26

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Bacteriophage T4 Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Deborah M. Hinton, Ph.D.	Research Chemist	LBP, NIDDK
Others:	Mridula Sharma, Ph.D.	Visiting Fellow	LBP, NIDDK
	Roslyn March-Amegadzie, Ph.D.	Senior Staff Fellow	LBP, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Nucleic Acid Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:

3.5

PROFESSIONAL:

3.0

OTHER:

.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been transferred to Z01-DK- 57802.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 23,900-02 LBP
PERIOD COVERED October 1, 1992, through September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Chemistry and Function of Microtubules		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Jan Wolff, M.D., Ph.D. Medical Officer and Chief, Section on Endocrine Biochemistry	LBP NIDDK
Others:	Dan Sackett, Ph.D. Expert Shobha Uppuluri, Ph.D. Visiting Associate Leslie Knipling Biologist Jay Varma Special Volunteer	LBP NIDDK LBP NIDDK LBP NIDDK LBP NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Endocrine Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
4.7	4.0	0.7
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Two colchicine-binding domains of <math>\beta</math>-tubulin have been identified by direct photolabeling, limited proteolysis with trypsin and chymotrypsin, immunoblot analysis, and sequencing of labeled peptides. The domains are (1) in the N-terminus starting at residue 1 and extending to residues 19-20 and possibly to residue 36; and (2) in the center of the monomer at residues 213/214 to 241/242. Thus, at least two domains of <math>\beta</math>-tubulin are involved in colchicine binding. The structural consequence of colchicine binding has been probed by limited hydrolysis with trypsin or chymotrypsin of the colchicine-tubulin complex; this yields a different proteolytic pattern than free tubulin. A new cleavage site appears with both enzymes: at Lys392 for trypsin and Phe389 for chymotrypsin. This structural change in the carboxy-terminal portion of the <math>\beta</math>-monomer may explain current models of substoichiometric inhibition of polymerization resulting from capping of the growing microtubule by a single layer of the colchicine-tubulin complex.</p>		





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,140-27 LBP

## PERIOD COVERED

October 1, 1992, through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Function of the Tryptophan Synthase Multienzyme Complex

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Edith Wilson Miles, Ph.D. Research Chemist  
and Chief, Section on Enzyme Structure and Function LBP NIDDK

Others: Syed A. Ahmed, Ph.D. Senior Staff Fellow LBP NIDDK  
Sergei Ruvinov, Ph.D. Visiting Fellow LBP NIDDK  
Utpal Banik, Ph.D. Visiting Fellow LBP NIDDK

COOPERATING UNITS (if any) Drs. D.R. Davies, C.C. Hyde & K. Parrish, LMB, NIDDK; C.C. Hyde, LSBR, NIAMS; P. McPhie, LBM, NIDDK; A. Ginsburg & D.P. Rameta, LB, LHLBI; P. Brzovic & M.F. Dunn, Univ. of California, Riverside; A. Mozzarelli & G.L. Rossi, Univ. of Parma, Italy; K. Yutani, Osaka U., Japan; K.S. Anderson, Yale Univ., New Haven, CT; R.S. Phillips, Univ. Georgia, Athens; D.L. Sackett, LBP, NIDDK; & A. Minton, LBP, NIDDK

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Enzyme Structure and Function

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL STAFF YEARS:

4.5

## PROFESSIONAL:

4.0

## OTHER:

0.5

## CHECK APPROPRIATE BOXES

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.) We are using the tryptophan synthase multienzyme  $\alpha_2\beta_2$  complex as a model system for investigating how protein-protein interaction and protein-ligand interaction affect enzyme structure and function. Our previous X-ray crystallographic studies of the wild type  $\alpha_2\beta_2$  complex from *Salmonella typhimurium* reveal that the active sites of the  $\alpha$  and  $\beta$  subunits are 25 Å apart and are connected by a tunnel. Thus reciprocal communication between the active sites of the heterologous subunits occurs over 25 Å and must involve protein conformational changes. We are using the crystal structure as a framework for selecting enzyme residues for site-directed mutagenesis. During the past year we have used several experimental techniques to investigate intersubunit communication and putative conformational changes in the wild type and mutant enzymes. (1) X-ray crystallographic studies have refined the structures of the wild type  $\alpha_2\beta_2$  complex and of two mutant forms and have detected conformational changes upon ligand binding. (2) Steady state and rapid kinetic studies have shown the four mutations in the  $\alpha$  subunit prevent a conformational change that affects channeling and intersubunit communication. Mutations in the interaction site between the  $\alpha$  and  $\beta$  subunits alter subunit association and intersubunit communication. (3) Differential scanning calorimetry is clarifying the effects of ligands and of subunit association on thermal unfolding. (4) Fluorescence spectroscopy has shown that  $\alpha$  subunit ligands and limited proteolysis of loop 6 in the  $\alpha$  subunit alter intersubunit communication and the interaction of Nile Red with a hydrophobic site within the  $\beta$  subunit in the  $\alpha_2\beta_2$  complex. (5) Spectroscopic studies have elucidated the mechanistic roles of  $\beta$  subunit Lys-87 in pyridoxal phosphate-dependent reactions. Lys-87 serves critical roles in transamination, catalysis and product release. Addition of ammonia to an enzyme-serine intermediate results in "chemical rescue" of the mutant enzyme and results in the formation of a stable aminoacrylate intermediate at the active site of the  $\beta$  subunit. Kinetic studies show that the aminoacrylate intermediate is the key intermediate which triggers the activation of the  $\alpha$  subunit.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PERIOD COVERED

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Others:	Dan Sackett, Ph.D.	Expert	LBP NIDDK
	Leslie Knipling	Biologist	LBP NIDDK

COOPERATING UNITS (if any)

## SECTION

#### INSTITUTE AND LOCATION

**TOTAL STAFF YEARS:**

CHECK APPROPRIATE BOX(ES)

**SUMMARY OF WORK** (Use standard unreduced type. Do not exceed the space provided.)



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK24,942-01 LBP
PERIOD COVERED October 1, 1992, through September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  PI:            Peter McPhie, Ph.D.                      Research Chemist                      LBP NIDDK		
COOPERATING UNITS (if any) Dr. W. Jakoby, LBM; Drs. J. Cheng and S. Adhya, LMB, NCI; Dr. V. Ivanov, Engelhart Institute of Molecular Biology, Moscow		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Enzyme Structure and Function		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS:  <div style="text-align: center;">1.0</div>	PROFESSIONAL:  <div style="text-align: center;">1.0</div>	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;">           (a) Human subjects                (a1) Minors                (a2) Interviews         </div> <div style="width: 30%;">           (b) Human tissues         </div> <div style="width: 30%;">           ✓ (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             This laboratory is engaged in studies on protein structure and the mechanism of protein folding. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight= 39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of its first 44 amino acids, to produce an enzymatically active protein, pepsin. Pepsin is stable only at pH's below 6. Pepsin and pepsinogen are unfolded by exposure to high pH, temperature or concentrations of denaturants, such as urea. However, unfolded pepsinogen can refold to its normal structure, when returned to native conditions, whereas pepsin cannot. I am interested in the mechanism of this refolding reaction and how the difference in sequence influences the refolding of the two proteins. We have used techniques such as ultra-violet, circular dichroic and fluorescence spectroscopies, together with chemical modification and peptide chemistry, to characterize the structures of the native and unfolded species. We have used rapid kinetic techniques, such as stopped-flow and T-jump, to detect partly folded forms in the folding reaction; their structures have been partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.           </p>		



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 24,150-22 LBP

## PERIOD COVERED

October 1, 1992, through September 30, 1993

**TITLE OF PROJECT** (80 characters or less. Title must fit on one line between the borders.)

Noncovalent intermolecular interactions in Biochemistry

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Allen P. Minton, Ph.D. Research Chemist  
and Chief, Section on Physical Biochemistry LBP NIDDK

Others:	German Rivas, Ph.D.	Visiting Fellow	LBP NIDDK
	Saleh Darawshe, Ph.D.	Visiting Fellow	LBP NIDDK
	Jianqing Wu, Ph.D.	Visiting Fellow	LBP NIDDK
	Arun K. Attri, Ph.D.	Courtesy Associate	LBP NIDDK

## COOPERATING UNITS (if any)

K. Ingham, Holland Laboratory, American Red Cross

## LAB/BRANCH

Laboratory of Biochemical Pharmacology

## SECTION

Section on Physical Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

**TOTAL STAFF YEARS:**

**PROFESSIONAL:**

OTHER:

3.8

### 3.3

0.5

## CHECK APPROPRIATE BOXES

◆ (a) Human subjects    ◆ (b) Human tissues    ✓ (c) Neither

♦ (a1) Minors

- ◊ (a2) Interviews

**SUMMARY OF WORK** (Use standard unreduced type. Do not exceed the space provided.)

The calcium-linked hetero-association of human complement subcomponents C1r and C1s has been quantitatively characterized as a function of calcium concentration via the technique of tracer sedimentation equilibrium.

The role of macromolecular crowding in the regulation of cellular volume has been further explored.

A new technique for the detection of associations in mixtures of several protein components, called sedimentation equilibrium - quantitative polyacrylamide gel electrophoresis (SE-QPAGE), has been developed and tested on mixtures of up to four protein components.

Theoretical models have been proposed for the (i) effect of confining proteins to small pores of different shapes, and (ii) and the effect of weak nonspecific attractive interactions between surfaces and proteins upon various conformational and association equilibria.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 24-260-26

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enzymatic Mechanisms of DNA Replication: The Bacteriophage T4 System

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Nancy G. Nossal	Research Chemist	LBP, NIDDK
Others:	Lisa Hobbs, Ph.D.	IRTA Fellow	LBP, NIDDK
	Todd Capson, Ph.D.	Senior Staff Fellow	LBP, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemical Pharmacology

SECTION

Section on Nucleic Acid biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

TOTAL STAFF YEARS:

3.6

PROFESSIONAL:

3.0

OTHER:

.6

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

This project has been transferred to Z01-DK- 57801.



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b> Z01 DK 24,590-22 LBP
<b>PERIOD COVERED</b> October 1, 1992, through September 30, 1993		
<b>TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)</b> Structure and Interactions of Biologically Important Macromolecules		
<b>PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)</b>		
<b>PI:</b> Harry A. Saroff, Ph.D.	Research Chemist (Intermittent)	LBP NIDDK
<b>Other:</b> Elemer Mihalyi, M.D., Ph.D.	Special Volunteer	LBP NIDDK
<b>COOPERATING UNITS (if any)</b>		
<b>LAB/BRANCH</b> Laboratory of Biochemical Pharmacology		
<b>SECTION</b> Section on Physical Biochemistry		
<b>INSTITUTE AND LOCATION</b> NIDDK, NIH, Bethesda, Maryland 20892		
<b>TOTAL STAFF YEARS:</b> <div style="text-align: center;">1.7</div>	<b>PROFESSIONAL:</b> <div style="text-align: center;">1.0</div>	<b>OTHER:</b> <div style="text-align: center;">0.7</div>
<b>CHECK APPROPRIATE BOX(ES)</b> <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="text-align: center;">             (a) Human subjects              (a1) Minors              (a2) Interviews           </div> <div style="text-align: center;">             (b) Human tissues           </div> <div style="text-align: center;">             ✓ (c) Neither           </div> </div>		
<b>SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)</b>  Cooperative binding systems are being studied taking into account site or sub-unit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems, particularly those involving the action of fibrinogen and the processes of protein-DNA interactions.		

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 24,709-12 LBP
PERIOD COVERED October 1, 1992, through September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Polyamine Biosynthesis and Function</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Celia White Tabor, M.D. Medical Officer (Research)	LBP NIDDK
Others:	Herbert Tabor, M.D. Supervisory Medical Officer (Research) Chief, Section on Pharmacology, LBP; and Chief, Laboratory of Biochemical Pharmacology LBP NIDDK David Balasundaram, Ph.D. Visiting Associate LBP NIDDK Nobuko Hamasaki, Ph.D. IRTA LBP NIDDK	
COOPERATING UNITS (if any) Drs. R. B. Wickner and J. Dinman, GSE, LBP, NIDDK		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Pharmacology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
5.0	4.0	1.0
CHECK APPROPRIATE BOX(ES) (a) Human subjects      (b) Human tissues <input checked="" type="checkbox"/> (c) Neither (a1) Minors (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The polyamines putrescine, spermidine, and spermine are major cellular components of all living cells and have been shown to be involved in many systems related to growth and differentiation. Our current and older studies have been directed at learning how these polyamines are synthesized and regulated and their physiological function <i>in vivo</i>. As part of these studies we are currently concentrating on the biochemistry, regulation, and genetics of these amines in <i>Saccharomyces cerevisiae</i>. In our past studies, we prepared mutants in the biosynthetic pathways and showed that cells that were deprived of spermidine or spermine were unable to grow, to sporulate, or to maintain the virus-like RNA killer plasmids of yeast. Our current studies have demonstrated two additional <i>in vivo</i> functions: (1) Spermidine protects yeast from oxidative damage due to superoxide formation... (2) We have also shown an unusual and unexpected effect of spermidine on protein synthesis <i>in vivo</i>. With the use of a model system for the study of ribosomal frameshifting, described by Wickner and Dinman, and in collaboration with them, we have shown that polyamines are essential for the maintenance of the correct translational efficiency of +1 ribosomal frameshifting. Ribosomal frameshifting is of particular importance in the development of retroviruses and is known to be important in the "gag-pol" type of protein synthesis in the L-A dsRNA system of yeast studied by Dinman and Wickner and in the Ty transposon system. Other studies have been concerned with the mechanism of suppressor mutations that overcome mutations in the biosynthetic pathway for the amines and on the regulation of the steps in this pathway.</p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 24,940-20 LBP																																								
PERIOD COVERED October 1, 1992, through September 30, 1993																																										
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Yeast RNA Virology																																										
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">Reed B. Wickner, M.D.</td> <td style="width: 40%;">Medical Officer, USPHS</td> <td style="width: 10%;"></td> </tr> <tr> <td></td> <td>and Chief, Section on Genetics of Simple Eukaryotes</td> <td></td> <td>LBP NIDDK</td> </tr> <tr> <td>Others:</td> <td>Tsutomu Fujimura, Ph.D.</td> <td>Visiting Scientist</td> <td>LBP NIDDK</td> </tr> <tr> <td></td> <td>Yutaka Matsumoto, Ph.D.</td> <td>Staff Fellow</td> <td>LBP NIDDK</td> </tr> <tr> <td></td> <td>Jonathan D. Dinman, Ph.D.</td> <td>Staff Fellow</td> <td>LBP NIDDK</td> </tr> <tr> <td></td> <td>Rosaura P. C. Valle, Ph.D.</td> <td>Visiting Fellow</td> <td>LBP NIDDK</td> </tr> <tr> <td></td> <td>Juan Carlos Ribas, Ph.D.</td> <td>Visiting Fellow</td> <td>LBP NIDDK</td> </tr> <tr> <td></td> <td>Kathleen Carroll, Ph.D.</td> <td>IRTA Fellow</td> <td>LBP NIDDK</td> </tr> <tr> <td></td> <td>Daniel Masison, Ph.D.</td> <td>IRTA Fellow</td> <td>LBP NIDDK</td> </tr> <tr> <td></td> <td>Yasuyuki Otake, Ph.D.</td> <td>Guest Researcher</td> <td>LBP NIDDK</td> </tr> </table>			PI:	Reed B. Wickner, M.D.	Medical Officer, USPHS			and Chief, Section on Genetics of Simple Eukaryotes		LBP NIDDK	Others:	Tsutomu Fujimura, Ph.D.	Visiting Scientist	LBP NIDDK		Yutaka Matsumoto, Ph.D.	Staff Fellow	LBP NIDDK		Jonathan D. Dinman, Ph.D.	Staff Fellow	LBP NIDDK		Rosaura P. C. Valle, Ph.D.	Visiting Fellow	LBP NIDDK		Juan Carlos Ribas, Ph.D.	Visiting Fellow	LBP NIDDK		Kathleen Carroll, Ph.D.	IRTA Fellow	LBP NIDDK		Daniel Masison, Ph.D.	IRTA Fellow	LBP NIDDK		Yasuyuki Otake, Ph.D.	Guest Researcher	LBP NIDDK
PI:	Reed B. Wickner, M.D.	Medical Officer, USPHS																																								
	and Chief, Section on Genetics of Simple Eukaryotes		LBP NIDDK																																							
Others:	Tsutomu Fujimura, Ph.D.	Visiting Scientist	LBP NIDDK																																							
	Yutaka Matsumoto, Ph.D.	Staff Fellow	LBP NIDDK																																							
	Jonathan D. Dinman, Ph.D.	Staff Fellow	LBP NIDDK																																							
	Rosaura P. C. Valle, Ph.D.	Visiting Fellow	LBP NIDDK																																							
	Juan Carlos Ribas, Ph.D.	Visiting Fellow	LBP NIDDK																																							
	Kathleen Carroll, Ph.D.	IRTA Fellow	LBP NIDDK																																							
	Daniel Masison, Ph.D.	IRTA Fellow	LBP NIDDK																																							
	Yasuyuki Otake, Ph.D.	Guest Researcher	LBP NIDDK																																							
COOPERATING UNITS (If any) Alasdair Steven, LSB, NIAMS; Tim Baker, Purdue University; Craig Hyde, LSB, NIAMS																																										
LAB/BRANCH Laboratory of Biochemical Pharmacology																																										
SECTION Section on Genetics of Simple Eukaryotes																																										
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892																																										
TOTAL STAFF YEARS: 7.6	PROFESSIONAL: 7.1	OTHER: 0.5																																								
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																																										
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The L-A virus of the yeast <i>Saccharomyces cerevisiae</i> encodes its own major coat protein (Gag) and a multifunctional RNA polymerase - RNA binding protein (Pol) made as a Gag-Pol fusion protein formed by a -1 ribosomal frameshifting event identical in mechanism to that used by retroviruses of animal cells for a similar purpose. We have isolated host mutants that have increased levels of ribosomal frameshifting and several of these have lost ability to propagate the M<sub>1</sub> satellite RNA. The mutants define eight <u>mov</u> (maintenance of frame) genes. Three mutants are ts for growth showing different cell cycle arrest phenotypes.</p> <p>We have described an antiviral system determined by the six chromosomal <u>SKI</u> genes. We now have evidence that this system works by inhibiting the translation of viral messages, apparently recognizing them as viral by their absence of a 5' cap structure or a 3' polyA sequence.</p> <p>We have shown that a chimeric RNA - dependent RNA polymerase, made up of part of the L-A enzyme and part of the Sindbis virus polymerase, is capable of supporting the replication and transcription of the M<sub>1</sub> satellite dsRNA in yeast. This supports the notion that our findings about the genetics of viral replication in yeast are applicable to mammalian viruses.</p> <p>We have evidence [in collaboration with the groups of Alasdair Steven, LSB, NIAMS, and Tim Baker, Purdue U.] that the structure of L-A virus particles is icosahedral with T=1 and an assymetric unit consisting of a dimer of Gag protein molecules.</p>																																										

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 24, 941-02 LBP
PERIOD COVERED October 1, 1992, through September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Membranes Cytoskeleton and Secretion		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Jan Wolff, M.D., Ph.D. and Chief, Section on Endocrine Biochemistry	Medical Officer  LBP NIDDK
Others:	Dan Sackett, Ph.D. Leslie Knipling	Expert Biologist LBP NIDDK LBP NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Endocrine Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS:	PROFESSIONAL:	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <div style="text-align: center; padding-top: 50px;">           Inactive in 1993 but has been restarted.         </div>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK24,942-01 LBP
PERIOD COVERED October 1, 1992, through September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  <div style="display: flex; justify-content: space-between;"> <span>PI: Peter McPhie, Ph.D.</span> <span>Research Chemist</span> <span>LBP NIDDK</span> </div>		
COOPERATING UNITS (if any) Dr. W. Jakoby, LBM; Drs. J. Cheng and S. Adhya, LMB, NCI; Dr. V. Ivanov, Engelhart Institute of Molecular Biology, Moscow		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Enzyme Structure and Function		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS: 1.0	PROFESSIONAL: 1.0	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-around; align-items: flex-start;"> <span>(a) Human subjects</span> <span>(b) Human tissues</span> <span>√ (c) Neither</span> </div> <div style="margin-left: 20px;">             (a1) Minors              (a2) Interviews           </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             This laboratory is engaged in studies on protein structure and the mechanism of protein folding. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight= 39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of its first 44 amino acids, to produce an enzymatically active protein, pepsin. Pepsin is stable only at pH's below 6. Pepsin and pepsinogen are unfolded by exposure to high pH, temperature or concentrations of denaturants, such as urea. However, unfolded pepsinogen can refold to its normal structure, when returned to native conditions, whereas pepsin cannot. I am interested in the mechanism of this refolding reaction and how the difference in sequence influences the refolding of the two proteins. We have used techniques such as ultra-violet, circular dichroic and fluorescence spectroscopies, together with chemical modification and peptide chemistry, to characterize the structures of the native and unfolded species. We have used rapid kinetic techniques, such as stopped-flow and T-jump, to detect partly folded forms in the folding reaction; their structures have been partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.           </p>		



## **Annual Reports of the Laboratory of Chemical Biology National Institute of Diabetes and Digestive and Kidney Diseases**

The Laboratory of Chemical Biology conducts research on molecular biology and genetics, especially as related to genetic diseases, and on the structure, function and dynamics of proteins. A major emphasis of the Laboratory is in understanding the molecular processes involved in the developmental control of the expression of the human hemoglobin genes. As part of this work, extensive studies on the pathophysiology of genetic diseases of hemoglobin and new approaches to their treatment have been developed. A second major emphasis of the Laboratory is in the study of forces that stabilize globular proteins. A third program concentrates on cytogenetic analysis of patients with genetic illnesses. Other new research initiatives include the study of transcriptional control of globin genes by silencers and the cloning and characterization of the human erythropoietin receptor gene. The protocol for treatment of sickle cell disease patients with hydroxyurea to elevate fetal hemoglobin has been enlarged to include protocols with the addition of recombinant human erythropoietin and also the treatment of patients with the thalassemia syndromes. A facility for the production of transgenic mice has been established.

The Laboratory has three Sections. The Section of Protein Chemistry and Conformation, under Dr. Hiroshi Taniuchi, is devoted primarily to studying the folding and assembly of globular proteins, especially cytochrome c. The Section on Molecular Forces and Assembly is the home of the Cytogenetics Unit under Dr. Beverly White, which is a joint endeavor of the Inter-Institute Genetics Program of the Clinical Center and NIDDK. The Section on Molecular Biology and Genetics, under Dr. Alan N. Schechter, is concerned primarily with the molecular basis of the developmental control of gene expression, especially in human erythroid cells, and its relevance to the understanding of the molecular basis of genetic diseases of hemoglobin and the red blood cell and approaches to their therapy. A Unit on Molecular Hematology, under Dr. Griffin Rodgers, exists in this Section to facilitate studies of the hemoglobin diseases.

During this last year, several personnel changes have occurred. The Laboratory is in the process of establishing new Sections for Drs. Griffin P. Rodgers and Constance Noguchi. Dr. Eitan Fibach of the Hadassah Medical School in Jerusalem, Israel has established an extensive series of collaborations, in his role as a Visiting Associate, between his home institutions, the Laboratory of Chemical Biology, and many other research groups at NIH, in the U.S. and abroad. Similarly, Dr. Shu-zhen Huang, a Visiting Associate, has established extensive interactions between this Laboratory and the Shanghai Institute of Medical Genetics. Dr. Setsuo Hasegawa, a Special Volunteer, is creating similar interactions with the Department of Hematology at Nippon Medical School and Dr. Steven Shapiro, also a Special Volunteer, is working to create interactions with the U.S. Department of Agriculture Research Station in Beltsville, Maryland for the production of learge animal transgenic models of human disease. Dr. Christian B. Anfinsen remains as a Scientist Emeritus in the Laboratory of Chemical Biology.

The other collaborations and work of the Laboratory of Chemical Biology are specified in each of the following individual project reports.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK25011-18 LCB

## PERIOD COVERED

October 1, 1992 to September 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Protein Folding

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Hiroshi Taniuchi Chief, Section on Protein Chemistry LCB, NIDDK  
and Conformation

Others: Alice Hawley Chemist LCB, NIDDK  
Greg Charles Biological Aid LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Protein Chemistry and Conformation

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 2.46

PROFESSIONAL: 1.8

OTHER: 0.66

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A long range goal of this project is to predict the three-dimensional structural of proteins based on the amino acid sequences. For this understanding of major interatomic interactions which stabilize the structure is necessary. Our recent studies have shown that the cytochrome c fragment complex is stabilized by the core domain-domain interaction. A core domain consists of a hydrophobic core and the surrounding shell and folds and unfolds as a unit. Our studies have suggested that this core domain-domain interaction is mediated by the residue-residue interaction in the ordered hydrophobic core which is called the core group interaction. To know more about this interaction effect of the Leu 32 to Nva and Leu 35 to Nva substitutions and a combination of them on the stability of a horse cytochrome c three-fragment complex have been studied. The complex (1-25)H.(28-38).(39-104) contains a heme fragment of residues 1 to 25, (1-25)H and two apofragments (28-38) and (39-104). It resembles the native protein with the exception that residues 39 to 55 are flexible. The Leu 35 to Nva did not significantly decrease or only slightly decreased the apparent equilibrium constant K of (28-38) with ferric complex (1-25)H.(39-104) at 15 degree and somewhat increased that with ferrous (1-25)H.(39-104) (Kred). It did not change heat stability of the 695 nm band of ferri (1-25)H.(28-38).(39-104), a band indicative of the Met80-S-Heme-Fe bond which is located on the left side of the heme as shown by Dickerson and Colleagues. In contrast, the Leu 32 to Nva decreased K and Kred, respectively by about 20 and 45 fold. It markedly reduced heat stability of the 695 nm band (about 0.7 kcal/mol). Perturbation of these properties by a combination of the two substitutions is similar to that by the Leu 32 to Nva alone. The rate constant for dissociation of fragment (39-104) of complex ferro- (1-25)H. (28-38).(39-104) (k), which occurs without going through ferro-(1-25)H.(39-104), was measured by the fragment exchange technique. The Leu 32 to Nva and Leu 35 to Nva, respectively increased k by 48 to 78 and 1.4 to 2.2 fold at 15 degree. Assuming that the positions of Leu 32 and Leu 35 are homologous to native cytochrome c, analysis of the data suggests that the Leu to Nva markedly perturbs a spatially long-range non-covalent interaction which exists in the ordered hydrophobic core of complex (1-25)H.(28-38).(39-104). This interaction involves gamma-methyl group of Leu 32 and the Fe-S bond and stabilizes the structure on both left and right side of the heme.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25016-19 LCB

## PERIOD COVERED

October 1, 1992 to September 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Trans-Acting Factor (s) Controlling Globin Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Alan N. Schechter

Chief

LCB, NIDDK

Others: Constance T. Noguchi

Research Physicist

LCB, NIDDK

Zi Yao Liu

Visiting Associate

LCB, NIDDK

Panagoula Kollia

Visiting Fellow

LCB, NIDDK

Betty Peters

Special Volunteer

LCB, NIDDK

## COOPERATING UNITS (if any)

Connaught Laboratories, Toronto, Canada, (Dr. S. X. Cao); Petah Tikva Hospital, Israel (Dr. M. Mittelman); University of Maryland (Dr. S. Shapiro)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 0.3

PROFESSIONAL: 0.3

OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have been studying the molecular genetic mechanisms that control the developmental switch from embryonic to fetal to adult hemoglobins with respect to their basic biology and their relevance to developing new therapies for diseases of hemoglobin. To study these processes we have been using two model systems: the K562 erythroleukemic cells and transgenic mice.

K562 is an erythroleukemic cell line used for the last decade as a model for the study of the control of the human globin gene expression. These cells do not support transcription of beta-globin gene but do express transcripts of epsilon and gamma-globin genes at very high levels when exposed to a number of inducing agents. Results from this and other laboratories suggest that the control of this pattern of expression is mediated by the presence and/or absence trans-acting factors which exert their action on sequences corresponding to the promoters of these genes. In the last few years transgenic mice have become an excellent model system for studying globin gene expression, and are supplementing the use of human cell lines.

We have previously reported the presence of a transcriptional control element with properties of a silencer extending from -392 to -177 bp relative to the cap site of the human epsilon-globin gene. Using deletion mutants and synthetic oligonucleotides in transient expression assays, DNA sequences responsible for this effect have been further delimited to 44 nucleotides located between -294 and -251bp. Gel electrophoresis mobility shift assays and DNase footprinting assays demonstrate that these negative regulatory sequences are recognized differently by proteins present in nuclear extracts obtained from HeLa and K562 cells. The protein present in K562 cells, but not in HeLa cells, that interacts specifically with this silencer binds to the same sequence recognized by the yeast binding protein ABF1.

We have initiated studies using the transgenic technique to study the epsilon-globin silencer, constructs with the silencer sequence intact, as well as constructs with the silencer mutated, have been injected into fertilized mouse ova to study the function of the silencer in this assay system. The results should help clarify the molecular mechanisms of silencing, an important aspect of hemoglobin study.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

ZO1 DK 25021-18 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sickle Cell Anemia: The Intracellular Polymerization of HbS

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Constance Tom Noguchi Research Physicist LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK  
 Alan N. Schechter Chief LCB, NIDDK

## COOPERATING UNITS (If any)

BEIB, NIH (R. Chadwick); Howard University (W. Poillon), Pennsylvania State University (C. Dong), George Washington University (L. Lessin), Paris Consortium (M. Redelsburger, R. Girot, J. Eliou), Nippon Medical School, Tokyo, Japan (N. Uyesaka).

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, MD

TOTAL STAFF YEARS 1.0

PROFESSIONAL: 1.0

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

The polymerization of sickle hemoglobin arises from the reduced solubility of concentrated deoxygenated sickle hemoglobin found within intact red blood cells from individuals with sickle cell anemia. We have examined the solubility of hemoglobin S mixtures with hemoglobins A, A2 and F at varying oxygen saturations to determine the extent of hemoglobin S polymerization under physiologic conditions. A detailed analysis of the solubility of mixtures of hemoglobin at various oxygen saturations provides the means to predict the maximum extent of polymerization within the sickle hemoglobin containing erythrocyte.

Ultracentrifugation of polymerized hemoglobin mixtures at different ligand concentrations was used to measure directly the equilibrium solubility of sickle hemoglobin mixtures. These measurements indicated that the solubility increases with ligand concentration and the critical oxygen saturation above which no polymer is detected is shifted to lower ligand concentrations as the percent fetal hemoglobin increases. The sparing effect of hemoglobin A2 was comparable to the sparing effect of hemoglobin F. Hemoglobin A and hemoglobin C exhibited similar effects on hemoglobin S polymerization in comparable mixtures. The current study also demonstrates explicitly the polymerization behavior of hemoglobin S and hemoglobin F mixtures at increasing ligand concentrations.

The theoretical model based on experimental data of polymerization of hemoglobin mixtures was used to analyze observations obtained from 2674 individuals with sickle cell anemia from the Cooperative Study of Sickle Cell Disease database. These data indicate that during the first twelve years of life, fetal hemoglobin decreased while mean corpuscular hemoglobin concentration increased until adolescence. These changes result in a continuous rise in polymerization tendency during the first twelve years of life. Data obtained from the Parisian Prospective Study on Sickle Cell Disease was used to determine the variation in fetal hemoglobin and polymerization tendency in children with sickle cell anemia during the first two years of life. These data indicated that 3 of 21 children had a significantly greater predicted polymerization tendency due to early decreases in hemoglobin F. These individuals will be studied prospectively to ascertain the relationship among polymerization tendency and various clinical manifestations of sickle cell disease.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25025-16 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Mechanism of Antigen-Antibody Interaction

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

<b>PI:</b> Hiroshi Taniuchi	Chief, Protein Chemistry and Conformation Section	LCB, NIDDK
<b>Others:</b> Paolo Rizzo	Visiting Fellow	LCB, NIDDK
Mona Shah	Student Volunteer	LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Protein Chemistry and Conformation Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.49

## PROFESSIONAL:

1.2

## OTHER:

0.29

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our previous studies of hydrogen exchange of monoclonal anti-yeast iso-i-cytochrome c (mAbs) have indicated that binding of the antigen to the mAb stabilizes the domains of the Fab fragment which are remote from the antigen-binding site. The Fab consists of the light chain (VL and CL, the variable and constant regions) and the VH and CH1 (the variable and the first constant region) of the heavy chain. In the studies of protein folding (see another report) we have advanced a hypothesis that the protein structure is stabilized by the residue-residue interaction associated with the ordered hydrophobic core which is called the core group interaction. Based on these we speculate that the hypothetical core group interaction associated with the VL-VH interface and the VL and VH cores may influence the interaction at the antigen-antibody interface.

To test this hypothesis, we wish to substitute the residues of the VL-VH interface and the VL and VH cores and determine if the substitution changes the affinity of the Fab to the antigen. To begin this study, we have sequenced cDNAs encoding VL and VH of three anti-yeast iso-i-cytochrome c mAbs 4-74-6, 2-96-12 and 4-128-6. The exception is that the sequence of 26 nucleotides at the 5' end was not determined for mAbs 4-74-6 and 4-128-6. Then, the amino acid sequences were deduced.

It is striking that the amino acid sequences of VL and VH of mAb 4-74-6 including complementarity determining regions (CDRs) are significantly more homologous to anti-lysozyme HyHEL5 (Sheriff, S. et al. (1987) PNAS **84**, 8075) than mAb 2-96-12. This is true despite the fact that the epitopes of mAbs 4-74-6 and 2-96-12 are closely related to each other as shown in the previous studies. Thus, the present results confirm the current idea that substitution and insertion of a limited number of residues of CDRs change the specificity of antigen recognition. Homology modeling of the VL-VH dimers has allowed us to assign the residues of the VL-VH interface and the VL and VH cores. Furthermore, it has suggested the following possibility. The overlapping epitopes of mAbs 4-74-6 and 2-96-12 may share the structural element consisting of Asp60-Glu61. This shared epitope element may interact with homologous structural elements of the antigen-binding sites of these two mAbs consisting of arginine and serine. These elements of the antigen-binding sites may be recognizable only in the three-dimensional structure but not the amino acid sequences of the CDRs.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 25028-14 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Development of Non-Invasive Methods to Assess Sickle Cell Patients

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Griffin P. Rodgers

Chief, Molecular Hematology Unit

LCB, NIDDK

Others: Hiroyuki Hiruma

Visiting Fellow

LCB, NIDDK

Alan N. Schechter

Chief

LCB, NIDDK

## COOPERATING UNITS (if any)

Clinical Hematology Branch, NHLBI (A.W. Nienhuis); Transfusion Medicine, CC (H. Klein); BEIB (Eli Walker); Biometry Branch, NEI (M. Podgor); MRC Laboratory, Kingston, Jamaica (G. Serjeant); Nippon Medical School (Dr. N. Uyesaka)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Unit on Molecular Hematology

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

## TOTAL STAFF YEARS

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been temporarily suspended; it will be resumed in the next reporting period.





## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25058-07 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Laboratory Models of Globin Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: David Ebb Special Volunteer LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK  
 In-Hoo Kim Visiting Fellow LCB, NIDDK  
 Kay Chin Chemist LCB, NIDDK

## COOPERATING UNITS (if any)

Children's National Medical Center, Washington, D.C. (Dr. Ebb)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, MD

TOTAL STAFF YEARS 1.3

PROFESSIONAL: 1.2

OTHER: 0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

We have been investigating the molecular mechanisms which govern the tissue and developmental stage-specific expression of the beta-like globin genes. Our efforts have focused on establishing the combination of trans-acting factors and DNA cis-elements that determines the low level of expression of the adult beta-like globin genes in immature erythroid tissues. Our current studies of hemoglobin ontogeny have employed the K562 erythroleukemia cell line as a tissue culture model of globin gene expression. Previous studies of this cell line revealed an embryonic-fetal phenotype, which is unique for low level expression of the delta globin gene in the absence of beta globin transcription. This phenotype contrasts markedly with normal erythrocytes from all stages of development, where expression of beta globin far exceeds delta, ranging from a ratio of 75:1 in fetal tissues to 40:1 in normal adults. Using transient expression assays, we have been exploring the possibilities that both the reversal of normal relative levels of synthesis of delta and beta globin, and the overall low levels of expression of the two adult genes in K562 cells are due to the differential binding of trans-acting proteins to upstream regulatory regions. DNA from the 5' flanking regions of the delta and beta globin genes was cloned into the expression vector PSVoCAT, then transfected into K562 cells. Our findings clearly demonstrate the existence of a negative transcription element in the -500 to -250bp region of the beta globin gene, consistent with previous studies by Berg, et al. (N.A.R., 1989), which identified 2 regulatory elements (BP-1 and BP-2) 5' to the beta globin gene. We have also identified an important positive transcription element in the comparable region flanking the delta globin gene. Structural analysis suggests that a potential regulatory element with extensive homology to the beta globin BP-2 motif exists in the delta globin 5' flanking region. We suggest that the negative regulatory elements upstream from the beta globin gene play an important role in repression of the adult beta-like globin genes in immature erythroid tissues.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25061-18 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression of Human Erythropoietin Receptor Gene in Transgenic Mice

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Z.Y. Liu

Visiting Associate

LCB, NIDDK

Others: Constance T. Noguchi

Research Physicist

LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.1

PROFESSIONAL: 1.1

OTHER:

## CHECK APPROPRIATE BOX(ES)



(a) Human subjects



(b) Human tissues



(c) Neither

☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Erythropoietin (Epo) is a primary regulator of erythropoiesis and functions by binding to the receptor (EpoR) on the surface of hematopoietic progenitor cells, followed by receptor-mediated endocytosis of the Epo molecule.

We have previously cloned and sequenced the human EpoR gene making it possible for us to examine the tissue and developmental specificity of gene expression in normal and transgenic mice. After generating several lines of transgenic mice expressing the EpoR, hematological parameters were measured in transgenic mice and tissue expression was determined in both normal and transgenic from different lines. Our results show that hematological parameters were within normal limits except for a suggestion of increased reticulocyte levels. Human EpoR transcripts were detected in erythroid tissues (bone marrow and spleen) and in the adult brain of transgenic mice. Based on the above discovery, we further examined developmental specificity of EpoR gene in mice. In normal mice, mouse EpoR transcripts in fetal liver were detected at 12 days and decreased with age, disappearing before birth. Endogenous transcripts were also detected in fetal brain. The transcripts at different developmental steps were quantized by competitive PCR. We found that EpoR mRNA was exponentially down-regulated upon development in fetal liver. In transgenic mice, copy number was detected by competitive PCR and tissue expression of the human EpoR transgene, was copy number dependent.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25063-07 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of Hydroxyurea on Fetal Hemoglobin Synthesis

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Arielle Boulet Special Volunteer LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK

## COOPERATING UNITS (if any)

Department of Biochemistry, Rockefeller University y (J. Manning), Division of Hematology, Cornell University Medical College (B. Weksler)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 0.6

PROFESSIONAL: 0.6

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Recent clinical trials have substantiated hydroxyurea (HU) to be a potent effector of hemoglobin F (HbF) production in patients with sickle cell disease. This augmentation in HbF levels in patients is associated with a decrease in relative beta synthesis and thus an inhibitory effect of HbF. In order to gain insight into the possible genetic mechanisms underlying these effects, we used cation exchange (CE) and reverse-phase high performance liquid chromatography (HPLC) to examine changes in the levels of each hemoglobin species. Samples from ten patients treated with HU were analyzed approximately twice each week. Within the first 50 days of HU treatment, HbF increased on average from 3.3% to 7%. The patients had an average initial ratio G-gamma/A-gamma of 0.72 which did not change with treatment. At baseline, approximately 15-20% of the fetal hemoglobin exists in the acetylated form (F1) as determined by CE-HPLC, independent of subtype. With HU treatment, the total amount of F0 and F1 increases roughly proportional to the increase in F-reticulocyte numbers. Although the total HbA2 remained constant during treatment, there was an increase in a fraction which migrated with HbA2. In patients treated with combination HU and erythropoietin, we found a further increase in both F1 and this modified HbS fraction to levels approaching 5% and 10%, respectively.

Subsequently, analysis of this modified beta-globin polypeptide showed that the NH2 was not blocked (Edman sequencing) and that the modifier had a mass of 300 close to the one of glutathione (307) (mass spectroscopic analysis). A dual strategy using deblocking and synthetic methods has been used followed by IEF analysis and confirmed the glutathione nature of the adduct. Chemically modified HbS may interfere with HbS polymerization and thus have an added clinical benefit acetylation of other proteins (e.g. histones) may be involved in the regulation of gene expression; naturally occurring glutathione adducts have only been reported very recently. We have subsequently used this unique property of sickle hemoglobin - glutathione adducts at Cys-93(B)-to examine the function properties of a recombinant sickle hemoglobin. It is possible that their formation is related to metabolic effects of HU treatment or, as with acetylation, this modification may be indicative of induced fetal erythropoiesis.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25064-07 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cytogenetic Investigations of Patients with Genetically Determined Disorders

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

<b>PI:</b>	Beverly J. White	Director, Cytogenetic Unit	LCB, NIDDK
<b>Others:</b>	Damrong Wangsa	Photographer, Scientific Technical	OD, CC
	Flanagan Whitsitt	Medical Technician	OD, CC
	Cynthia Powell	Clinical Associate	OD, CC

## COOPERATING UNITS (if any)

Medical Genetics Programs, CC (W. Gahl, D. Parry); DE, NICHD (L. NELSON); LN, NIA (N. Shapiro, D. Murphy, W. Rapoport); Dept. Molecular Biology & Genetics, Wayne State University School of Medicine, Detroit (R. T. Taggart).

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Molecular Forces and assembly (Cytogenetics Unit)

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL MAN-YEARS: 4.5

PROFESSIONAL: 4.0

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The unit provided cytogenetic services for NIH patients, participated in the Interinstitute Medical Genetics Training and College of American Pathologists proficiency testing programs, responded to public inquiries, and collaborated in research projects. The patient database of 1,300 includes many with short stature, premature ovarian failure (POF), Turner syndrome (TS), endocrine disorders, mental handicap, recognized syndromes, and neurological disease. Our studies were requested to determine protocol eligibility, identify genetic variants, diagnose syndromes, and detect aberrations to localize disease genes.

Controlled studies of rRNA gene activity in Alzheimer's disease and normal aging were reported. Variations in structure and activity, measured by silver staining of chromosomal rDNA and protein (NORs), were found to be not associated with disease or aging. We then studied cytogenetic variation in 116 TS patients; 35% were genetic variants and 65% typical nonmosaic 45, X. Fluorescent In-Situ Hybridization (FISH) with X- and Y-specific DNA probes was required for identification of 4 variants. Controlled CNS imaging and cognitive studies of 18 of the TS patients were reported; significant differences were found, and nonmosaics were more affected than mosaics. Of 153 POF patients, 5% were familial, and 2% had abnormal karyotypes, including one with an X; autosome rearrangement. Her molecular studies suggested a critical locus within proximal Xq. Probes from this and a more distal Xq critical region might detect microdeletions in karyotypically normal familial POF. We also evaluated autistic savants and childhood schizophrenics for dysmorphic features and karyotypic abnormalities. These were done to better define the disorders physically and detect aberrations for disease gene localization.

As medical genetics training and research at NIH expand. We expect to continue our clinical services, interaction with the medical genetics training program, and collaborative projects.

1871

Month	Jan	Feb	Mar	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec
1871	1	2	3	4	5	6	7	8	9	10	11	12



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25070-05 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analysis of the Epsilon Globin Gene Flanking Sequences

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Panagoula Kolliou Visiting Fellow LCB, NIDDK

Others: Kyung Chin Research Biologist LCB, NIDDK  
 Constance T. Noguchi Research Physicist LCB, NIDDK  
 Alan N. Schechter Chief LCB, NIDDK

## COOPERATING UNITS (if any)

Hadassah University Hospital (Dr. Eitan Fibach), Jerusalem, Israel

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.3

PROFESSIONAL: 1.2

OTHER: 0.1

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

The developmental switch in hemoglobin synthesis, from embryonic (including epsilon globin) to fetal (gamma) to adult (beta) hemoglobins, that occurs during ontogeny is of great interest because of its fundamental biology as well as its relevance to the treatment of hemoglobinopathies. We have been studying transcriptional regulation of globin genes to clarify the molecular mechanisms of this switch. Transcriptional activity of globin genes is determined by a variety of cis-acting regulatory DNA sequences and trans-acting proteins. One of the cis-acting elements is the silencer located in the region between -177 and -392bp 5' to the epsilon globin gene, which was characterized in this laboratory. To delineate the molecular mechanism of the silencer and its possible role in the developmental regulation of epsilon gene expression, silencing of the epsilon gene in human adult erythroid cells (hAEC) was studied. The K562 erythroleukemia cell line constitutively expresses low levels of embryonic and fetal, but not adult hemoglobin and can serve as a control. We have now shown that in hAEC the mRNA level of each exon of the epsilon gene is present in low and varied amounts but the full length epsilon message is not present. In contrast, in K562 cells, where epsilon-globin gene is expressed, the full length epsilon-message is detectable and the mRNA level among the three exons is high and similar. Detection of the other globin messages (gamma and beta) showed that in hAEC beta-mRNA exon levels are high and gamma-mRNA levels are low. In K562 cells beta-mRNA levels are very low. As expected, in these cells which express high levels of gamma-globin, gamma-mRNA levels are high. Moreover, all or the majority of the globin transcripts for the highly expressed globin genes in both cell types are correctly spliced in the cytoplasm and the nucleus. The globin-transcripts, detected in cells where are not expressed, are unspliced (all or part of the transcripts). The above results may indicate that during erythropoiesis when erythroid cells switch from expressing epsilon-to-gamma-globin and then to beta-globin gene, changes in the transcription rate of epsilon-transcripts lead to the suppression of epsilon-gene transcription. These changes may activate inappropriate RNA splicing mechanism, thus unspliced epsilon-transcripts are detected when epsilon-gene is silent. 5' flanking sequences of the epsilon-globin gene, like the epsilon-globin gene silencer may contribute to the above changes.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01-DK 25073-04

PERIOD COVERED

**October 1, 1992 to September 30, 1993**

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Erythropoietin Receptor and its Genetic control in Red Cell Development**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Donna Williams	Special Volunteer	LCB, NIDDK
Others:	Teresa A. Zimmers	Special Volunteer	LCB, NIDDK
	Eitan Fibach	Visiting Associate	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

COOPERATING UNITS (if any)

John Hopkins Univ., Baltimore, MD (Drs. W. David Hankins and Donna Williams) LMCB, NCI (Dr. J.H. Pierce)

LAB/BRANCH

**Laboratory of Chemical Biology**

SECTION

**Section on Molecular Biology and Genetics**

INSTITUTE AND LOCATION

**NIDDK, Bethesda, Maryland**

TOTAL STAFF YEARS:

**1**

PROFESSIONAL:

**1**

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**This project has been discontinued.**



PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism(s) of Enhanced Gamma Globin Gene Expression in Patients

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: In-Hoo Kim Visiting Fellow LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK  
Alan N. Schechter Chief LCB, NIDDK  
Eitan Fibach Visiting Associate LCB, NIDDK

COOPERATING UNITS (if any)

University of Maryland School of Medicine, Baltimore, Maryland (P. Berg)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section of Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.2 PROFESSIONAL: 1.2 OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Several lines of clinical and experimental evidence suggest that elevated levels of fetal hemoglobin (HbF) may improve the clinical course of individuals with sickle disease and  $\beta$ -thalassemia. A number of cytotoxic drugs have shown to enhance gamma-globin synthesis (and HbF levels) in animals and patients with hemoglobinopathies, although the mechanism of action of these is not known. The K562 human erythroleukemia cell line shows constitutively low levels of embryonic and fetal hemoglobin, and can be reversibly induced to increase gamma-globin gene expression in response to hydroxyurea and other agents. We are therefore using the K562 and human erythroid progenitor cells as a model system to understand the mechanism of induction of globin gene transcription. Both cells have been grown in the presence of 25mM hemin and 100mM hydroxyurea. By gel shift analysis, we show different mobility shift patterns at -226 to -134 of the g-gene promoter in the hemin and HU treated cells. This suggests the possibility of a novel binding activity underlying the hemin and hydroxyurea effect. The status of *in vivo* protein-DNA interactions in the promoter region of the Ggamma-gene was investigated by a new ligation-mediated polymerase chain reaction (LMPCR), using N-ethyl-N-nitrosourea (ENU). We find that ENU acts efficiently on suspension cells and can detect protein-DNA interactions not revealed by the commonly used dimethyl sulfate (DMS) method. *In vivo* footprinting results suggest that both CCAAT sites are activated in gamma-globin gene transcription, octamer binding site is specific for hemin induction, and -50 and -200 regions have major roles in gamma-globin transcriptional control. The protection pattern in the -200 region strongly suggests that repressor molecules bind in adult stage and activators in untreated and hemin treated K562 cells. Incubation in hydroxyurea inhibits binding at this region. It is hoped that further identification, characterization and purification of these putative binding proteins would not only extend the current knowledge of the molecular basis of the fetal to adult "switch", but also suggest novel pharmacological approach to the reversal of this switch in several clinically significant hemoglobinopathies.

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25076-04 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Characterization of the Epsilon-Globin Silencer

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Betty Peters

Robert Wood Johnson Fellow

LCB, NIDDK

Others: Constance T. Noguchi

Research Physicist

LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Molecular Biology and Genetics Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.1

## PROFESSIONAL:

1.1

## OTHER:

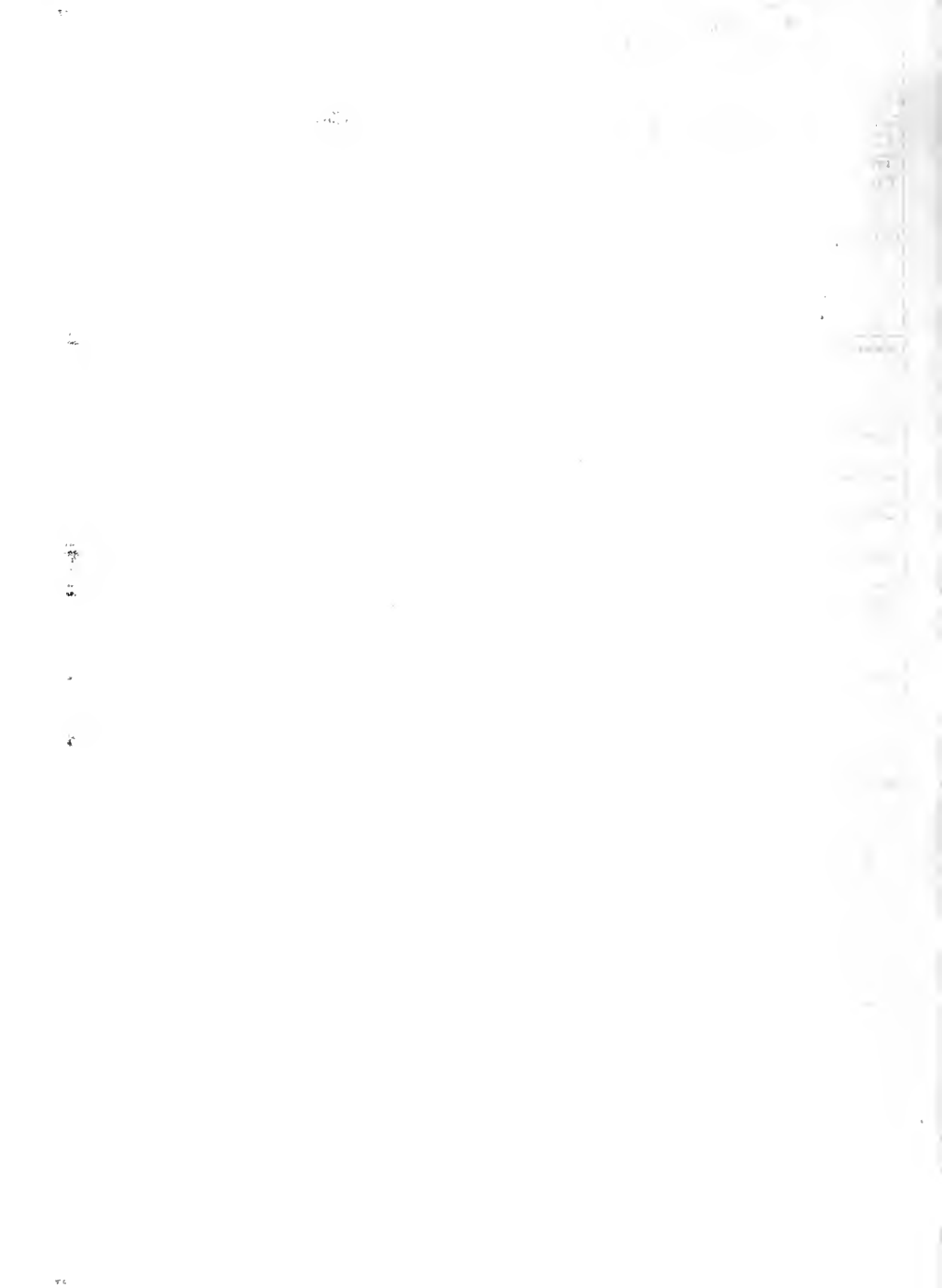
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## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Hemoglobin switching is an example of tissue specific and temporal regulation of gene expression. In humans, embryonic globin chains (zeta and epsilon) are expressed during the first trimester of gestation followed by a switch to the expression of fetal globin chains early in the second trimester and adult globin chains after birth. Previous work performed in the Laboratory of Chemical Biology has identified a silencer 200 to 400 bp upstream of the epsilon-globin gene which has been implicated in suppressing epsilon globin gene expression during the fetal and adult stages. Our work has focused on characterizing proteins binding to the silencer and the functional activity of the binding of trans-acting factors to the silencer. We are in the process of elucidating the mechanism(s) that trans-acting factors interacting with the silencer utilize in order to regulate epsilon-globin transcription. We are also studying the interactions of the silencer with other regulatory elements controlling epsilon-globin gene expression. Using DNase I footprinting, we have identified several sites of protein binding to the silencer. The major protected region shares a high percentage of homology with the binding sites of an erythroid specific transcription factor GATA-1 and a ubiquitous silencer binding protein, YY1. Using gel mobility shift assays with K562 nuclear extract, YY1 protein and a probe bracketing the major protected region and competitor DNA with mutations in the sites homologous to GATA-1 and YY1 binding sites we have demonstrated that a protein, likely GATA-1 binds to the GATA site in the silencer and that another protein, likely YY1, binds to the silencer. We are currently in the process of examining the activity of GATA-1 and YY1 on the expression of beta-like globin genes and determining the mechanism by which GATA-1 and YY1 binding to the silencer inhibits epsilon-globin gene expression.





PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Globin Gene Expression and the Treatment of Hemoglobinopathies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Schu-zen Huang Senior Staff Fellow LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK  
Alan N. Schechter Chief LCB, NIDDK  
Oui Shi Special Volunteer  
Min-he Hu Special Volunteer

COOPERATING UNITS (if any)

Shanghai Institute of Medical Genetics and Children's Hospital, Shanghai, China (Drs. Y.-T.Zeng, Zhao-rui Ren, Ying Huang and Mai-Jue Chen).

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.4

PROFESSIONAL: 1.4

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The beta-thalassemias are the commonest blood disorder resulted from a number of genetic defects in beta-globin gene expression. Previously, our group and others have shown that hydroxyurea (HU) can augment fetal hemoglobin synthesis in patients with sickle cell anemia, and, to a lesser degree, in patients with beta-thalassemia. However, the mechanism of the action remains unknown. Recently, we analysed the effects of daily lower dosage of HU administration in eight beta-thalassemia patients. Globin mRNA amounts and globin chain biosynthesis pre-and post-HU treatment were investigated by using our newly developed competitive, reverse transcriptase polymerase chain reaction (RT-PCR) and micro-biosynthetic test, respectively. Two patients showed an increase of at least 2-fold in Hb F levels and of 5-fold in gamma-globin mRNA. Curiously, two other patients with beta-thalassemia intermedia who were heterozygous for the IVS-2-654 C->T splicing mutation, with the other beta-allele normal from position -100 to the 3' poly A site, showed an obvious increase primarily in beta-globin biosynthesis, corresponding to an increase in beta-globin mRNA without a significant change in gamma globin synthesis. This was accompanied by an apparent clinical improvement and more effective erythropoiesis with an increase in absolute reticulocyte count and hemoglobin concentration. Thus, in addition to its effects in stimulating gamma-globin synthesis, HU may be useful in the context of treatment of beta-thalassemia through other mechanisms. Current studies in progress are aimed (1) to determine whether particular beta-thalassemia mutations are more susceptible to globin enhancing effects, (2) to define the unknown mutation or silent mutant sequences in the other beta-allele in the two patients who have shown an enhancement of beta-globin gene expression in these-homozygous and heterozygous beta-thalassemia individuals, using competitive RT-PCR method.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 25078-03 LCB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Globin Expression in an Erythroid Progenitor Culture System

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Eitan Fibach

Visiting Associate

LCB, NIDDK

Others: Constance T. Noguchi

Research Physicist

LCB, NIDDK

Griffin P. Rodgers

Chief, Molecular Hematology Unit

LCB, NIDDK

Alan N. Schechter

Chief

LCB, NIDDK

COOPERATING UNITS (if any)

Dvorit Samid Clinical Pharmacology Branch, NICI

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, MD

TOTAL STAFF YEARS

1.3

PROFESSIONAL:

1.3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Increased fetal hemoglobin (HbF) in cells of patients with beta hemoglobinopathies (beta thalassemia and sickle cell anemia) ameliorates the clinical symptoms of the underlying disease. Recently, several pharmacologic agents have been used to stimulate HbF synthesis; treatment of patients with 5-azacytidine or hydroxyurea result in higher HbF levels; yet, the clinical benefit is still unclear. Since these agents may be toxic and/or have been implicated in carcinogenesis, there is a considerable interest in finding other agents with the potential to increase HbF. So far only a handful of agents have been tested, mainly due to the lack of an appropriate experimental system that allows a rapid and accurate determination of the effect on relevant cells.

We have recently developed a novel, two-phase liquid culture system for growing erythroid progenitors derived from the peripheral blood of normal individuals and patients with hemoglobinopathies. We showed that the system recapitulates many hematological effects of hydroxyurea in vivo, including stimulation of HbF production. The purpose of our research is to utilize this procedure and modified methods for quantitation of hemoglobins developed by us lately to study the regulation of Hb production and for screening of agents for their HbF-stimulating potential with the final objective to produce an efficient, low toxicity therapy. The experiments include optimization of the conditions for stimulation of HbF production, followed by screening of various agents (including derivatives of hydroxyurea, phenyl-fatty acids, erythropoietin, hemin) and subsequently studying their mode(s) of action. The latter studies will provide the rationale for further screening of additional agents and for their chemical modification in order to increase efficacy. Classification of the drugs according to functional and mechanistic considerations will enhance experiments with combination of drugs belonging to different groups and, thus, expected to act synergistically.

In addition, we are attempting to develop, based on the cell culture system, an assay for evaluating an individual patient's response to a particular drug or drug combination. This will prevent both expensive and potentially risky treatment from patients which do not respond and suggest an alternative treatment (e.g. by other agents).

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## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25079-02 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Filterability of Mixtures of Sick and Normal Erythrocytes - Implication for Exchange Transfusion

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Setsuo Hasegawa Special Volunteer LCB, NIDDK

Others: Griffin P. Rodgers Chief, Molecular Hematology Unit LCB, NIDDK  
 Alan N. Schechter Chief LCB, NIDDK  
 Constance T. Noguchi Research Physicist LCB, NIDDK  
 Hiroyuki Hiruma Visiting Fellow LCB, NIDDK

## COOPERATING UNITS (if any)

Nippon Medical School, Tokyo, Japan (N. Uyesaka)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.3

PROFESSIONAL: 1.3

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

RBC transfusion has long been and currently remains an integral part of the management of sickle cell disease (SCD). The judicious use of blood can be both life-saving and life-prolonging in a variety of clinical settings. There are two broad indications for blood transfusion in SCD, maintenance of the oxygen-carrying capacity of the blood and dilution of the circulating, HbS containing cells. The latter may be accomplished both by the direct dilutional effect of transfused blood but also secondarily by suppression of the bone marrow and decreased production of cells capable of sickling.

Erythrocyte exchange offers numerous potential advantages over simple transfusion for the management of certain complications of SCD, especially if there is concern of causing circulatory overload or inducing a hyperviscosity state, which may actually exacerbate the propensity to sickle by retarding capillary transit. Guidelines for exchange transfusion therapy should be derived from an understanding of the rheological behavior of sickle cells. Microvascular occlusion by poorly deformable erythrocytes is believed to be the key pathophysiologic event in SCD. We, therefore, investigated the deformability of sickle (SS) cells and their mixture with normal (AA) cells in terms of filterability through nickel mesh. We also examined the role of dense SS cells and dense SS reticulocytes in overall deformability. In this study, we found that the filtration of mixtures of SS and AA erythrocytes was affected in almost linear fashion by both the %SS erythrocytes and %dense cells, and that dense cells impaired the filtration of these mixtures about 25 times as much as that of SS erythrocytes with no dense cells did. Filtration of mixtures of SS cells with no dense cells and AA cells of more than 60% was almost the same as that of normal erythrocytes. These results suggest that exchange transfusion therapy should aim at decreasing dense cells as much as possible, as well as at keeping the level of SS cells to the total cells below 40%. Our results also indicate the possibility that dense SS reticulocytes involved in reduced deformability. Our data, using an automated reticulocyte analyzer, showed that the proportion of high fluorescence-reticulocytes was elevated in the patients with SCD, which indicated the increased regeneration of immature reticulocytes. The benefit of exchange transfusion, therefore, can be not only due to direct dilution effect, but also due to suppression of SS reticulocytes. These studies can provide the practical guideline for exchange transfusion program based on the rheological behavior of sickle cells.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25080-02 LCB

## PERIOD COVERED

October 1, 1992 to September 30, 1993

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transcriptional Regulation of Human Erythropoietin-Receptor Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Naoko Oda

Visiting Fellow

LCB, NIDDK

Others: Constance Noguchi

Research Physicist

LCB, NIDDK

## COOPERATING UNITS (If any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS 1.4

PROFESSIONAL: 1.4

OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The hormone erythropoietin (Epo), which interacts with a high affinity cell surface receptor on the erythropoietin receptor (EpoR), developing erythroblasts is critical for normal erythroid development. Intracellular signaling consequent to binding of the ligand to its receptor provides a proliferative response and ensures differentiation of the erythroid lineage. We have recently cloned the genomic DNA of the human EpoR. In its 5' flanking region, there are potential regulatory sequences specific to the erythroid cell lineage. We have studied the tissue specificity of the expression of the hEpoR gene using transient transfection reporter gene assays and have also determined the extent that GATA-1, a transcription factor with erythroid specificity, can transactivate the hEpoR promoter.

We used transient transfection assays to examine the activity of the hEpoR promoter in human erythroleukemia cell lines, OCIM1 and K562, which express high and low levels of EpoR protein on the cell surface, respectively. Deletion analysis of the 5' flanking DNA of the hEpoR promoter linked to a luciferase reporter gene and transfected into OCIM1 and K562 cells indicate that much of the transcription activity is contained within a 150 bp proximal promoter. In HeLa cells, which do not express EpoR, transfected EpoR promoter was transactivated by cotransfection with a GATA-1 expression vector. Cotransfection with GATA-1 was also carried out in the erythroleukemia cells with constitutive levels of GATA-1. We observe that increasing GATA-1 levels in K562 cells can further increase EpoR promoter activity by 6 fold. In contrast, no significant increase was observed in OCIM1 cells in which the endogenous EpoR gene is already active. The levels of EpoR mRNA in OCIM1 cells is greater than in K562 cells. These results suggest that GATA-1 levels in OCIM1 may be saturating, and increasing GATA-1 expression has little effect on EpoR promoter activity and explain the dependency of EpoR expression which parallels the increase and decrease in GATA-1 levels during differentiation and maturation of erythroid progenitors. At the level of transcriptional activation, the interactions of GATA-1 or other transcriptional factors with the EpoR promoter should improve understanding of erythroid specific gene regulation.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 25081-01

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Covalent Modification of Hemoglobin in Hydroxyurea Treated Patients

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Arielle Boulet

Special Volunteer

LCB, NIDDK

Others: Griffin P. Rodgers

Chief

LCB, NIDDK

Molecular Hematology Unit

COOPERATING UNITS (if any)

Department of Biochemistry, Rockefeller Univ., (J. Manning), Division  
of Hematology, Cornell Univ. Medical College (B. Weksler)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:

.5

PROFESSIONAL:

.5

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been discontinued.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK-25082-02

PERIOD COVERED  
October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kyung Chin Microbiologist LCB, NIDDK

Others: Constance Tom Noguchi Research Physicist LCB, NIDDK

COOPERATING UNITS (if any)  
Beijing Medical College, Beijing, China (Dr. Y. Wu)  
Brown University (Dr. A. A. Anagnostou)

LAB/BRANCH Laboratory of Chemical Biology

SECTION Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION NIDDK, Bethesda, MD

TOTAL MAN-YEARS: 1.4 PROFESSIONAL: 1.4 OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Binding of erythropoietin (Epo) to the erythropoietin receptor (EpoR) on the surface of erythroid progenitor cells stimulates erythropoiesis resulting in molecular events including increases of GATA-1 protein, an erythroid specific transcription factor, and activation of globin genes. We have previously cloned and sequenced the human erythropoietin receptor gene. To examine the transcription control of gene expression, we have identified a 150 bp proximal promoter which contains binding sites for the general transcription factor, SP1, and the erythroid specific transcription factor, GATA-1, but no TATA or CAAT sequences generally associated with cellular promoters.

We use gel mobility shift assays with a DNA probe consisting of the erythropoietin receptor proximal promoter to demonstrate the ability of proteins to bind to the GATA-1 and SP1 consensus sequences. These data also indicate that proteins can bind to both sites simultaneously. That binding to these sites also occurs within the intact cell was supported by *in vivo* footprinting of the erythropoietin promoter region within the human erythroid cell line, OCIM1. *In vitro* footprinting also indicated proteins binding to an AP2 consensus sequence located in the region 5' to the GATA-1 binding site and another region located around -175 bp. Some protection in the region of expected AP2 binding could also be detected in the *in vivo* footprinting analysis.

Transient transfection assays linking deletions of the proximal promoter for the erythropoietin receptor with a luciferase reporter gene in OCIM1 and HeLa cells was used to examine the functional activity of DNA sequences capable of binding nuclear proteins. Reporter gene plasmids containing these deletion mutations indicated that SP1, GATA-1 and AP2 binding sequences modulate hEpoR promoter activity. Surprisingly, residual promoter activity was still present when sequences 5' (upstream) of the SP1 binding site including the GATA-1 sequence were deleted.

Co-transfection of the erythropoietin receptor promoter/luciferase reporter gene construct with an eukaryotic expression vector for GATA-1 indicated that GATA-1 is able to transactivate the erythropoietin receptor. Mutation of the GATA-1 site or SP1 site markedly reduced the erythropoietin receptor promoter activity in OCIM1 cells. Together, these data indicate that SP1 is required for activation of the erythropoietin receptor promoter and that GATA-1 likely contributes to the tissue specific expression of the erythropoietin receptor.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK-25083-01 LCB

PERIOD COVERED

October 1, 1992 to September, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Utilization of Triple Helical DNA as a Potential Means of Increasing Gamma Globin Expression in Adult

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Alphonso Brown

Howard Hughes Scholar

LCB, NIDDK

Others: Griffin P. Rodgers  
Eitan Fibach

Chief, Molecular Hematology Unit  
Visiting Associate

LCB, NIDDK  
LCB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Biology

SECTION

Section on Molecular Biology and Genetics

INSTITUTE AND LOCATION

NIDDK, Bethesda, MD

TOTAL STAFF YEARS 1.2

PROFESSIONAL: 1.2

OTHER: Medical Student

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The therapeutic benefits of increased gamma globin expression in the presence of hemoglobinopathies such as sickle disease are well documented. Unfortunately, the molecular control mechanisms which regulate the postpartum decrease in gamma globin levels are still not well understood. Recently it has been shown that certain unique DNA sequences have the ability to form triple helices with complementary third strands occasionally leading to alterations in the cognate gene expression. Triple helix formation, referred to as Hoogsteen base pairing, occurs between purine and pyrimidine nucleotides according to the following schemata, A-AT, T-AT, G-GC, C+-GC.

The uniqueness of potential triplex regions in native DNA as well as the increased binding stability over double stranded (ds) DNA has led to the development of triple helix forming oligonucleotides as site specific controls of gene expression. Several studies have shown that the formation of DNA triple helices at specific enhancer sites results in decreased expression of select target genes.

Our study focused on a 20 b.p. long purine rich region of DNA approximately 189 b.p. upstream from the gamma globin cap site. Based on prior evidence we believe this target region to have silencer activity which is activated by a repressor protein which binds in the region from -202 through -196 b.p. We have used specially designed triple helix forming oligonucleotides to serve as a site specific inhibitor of repressor protein binding in the -202 through -196 b.p. region of the gamma globin promoter. By inhibiting binding of the repressor protein we hoped to thereby increase the levels of gamma globin gene expression. A variety of in vitro and in vivo studies were used to assess the effect of triple helix formation in both the transformed K562 erythroleukemia cell line, and erythroid cells derived from progenitor cells in circulating mononuclear cells of normal individuals. We were able to successfully demonstrate in vitro triple helix formation within our target region in the gamma globin promoter. The in-vivo data is still being developed.



## ANNUAL REPORT OF THE LABORATORY OF CHEMICAL PHYSICS NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research in the Laboratory of Chemical Physics is primarily concerned with problems in structural biology and biophysical chemistry. There are direct applications of much of this work to the pathophysiology and treatment of human diseases such as AIDS, malaria, and sickle cell anemia. A variety of state-of-the-art structural and spectroscopic techniques are employed in these investigations, including nuclear magnetic resonance (NMR), Raman and infrared spectroscopies, time-resolved optical spectroscopy with nanosecond and picosecond lasers, and non-linear optical spectroscopy. There is also a major effort in theoretical studies to complement the experimental work, including both analytical methods and the use of high speed computers in large scale calculations. The systems under study include proteins, nucleic acids, intact and model membranes, retinal photoreceptors, muscle and small prototypical molecules for biological chromophores. Current research focusses on the development and application of new NMR methods to the determination of the three-dimensional structure of polypeptides and proteins in solution, the mechanism of protein folding, dynamics of ligand binding and conformational changes in proteins, conformational and dynamical properties of membrane systems, theoretical analysis of kinetics and chemical dynamics, computer simulations of protein motions, the analysis of excited electronic states of polyenes, the molecular mechanism of excitation in photoreceptor cells and ionic processes in cell membranes, the gelation of hemoglobin S and its relation to the pathophysiology and therapy of sickle cell disease, and the synthesis of potential therapeutic agents for malaria.

Bax and colleagues continue to make major advances in the development of new methods for the determination of the three-dimensional structure of proteins in solution. During the past year Bax and colleagues have developed a variety of new methods, including the determination of side chain rotamer conformations based on the measurement of the fraction of magnetization transferred from one nucleus to its partner by three-bond J couplings using  $^{13}\text{C}$ ,  $^{15}\text{N}$ , and  $^1\text{H}$  nuclei, the development of a powerful protocol based on  $\text{C}^\alpha$  and  $\text{C}^\beta$  chemical shifts to obtain sequential resonance assignments in medium sized proteins, and demonstration that the sequential assignment process can be facilitated by using uniform deuteration combined with high power deuterium decoupling to obtain narrowing of  $^{13}\text{C}$  resonances. They have also shown that it is possible to carry out a detailed structural analysis on detergent solubilized proteins without recourse to perdeuterated detergent. The method has been applied to calcineurin B, and has shown that the secondary structure of this protein is highly homologous to that of the smaller protein calmodulin. In recognition of their accomplishments in the nuclear magnetic resonance of proteins Ad Bax and Marius Clore shared the 1993 Protein Society Young Investigators Award.

Clore, Gronenborn and colleagues have developed and exploited multi-dimensional NMR techniques to determine the three-dimensional structures of a number of different proteins in solution and have developed new methods for assessing the quality, the limits of precision and the accuracy of the structures. During the past year the new structures solved by this group include the zinc-binding domain of the chicken erythroid transcription factor GATA-1 complexed to its cognate DNA site which is an unusual complex in that both the major and minor groove binding are involved, the human cytokine interleukin-4 from which it was possible to delineate





the receptor binding sites, and the immunoglobulin binding domain of Streptococcal G protein. Clore and Gronenborn have also used nuclear magnetic resonance to study the dynamics and folding of proteins. They have carried out the first NMR-folding study of an all  $\beta$ -sheet protein (interleukin-1 $\beta$ ) and found that stable secondary structures are formed much more slowly than in mixed  $\alpha$  helix/ $\beta$  sheet proteins.

Becker and colleagues have been exploring the mechanism and potential applications of the effect of isotopic substitution on NMR chemical shifts of nuclei up to 10 bonds away from the site of substitution. They have also been studying weak intermolecular interactions in liquid crystal solvents as a way of measuring very small distortions in molecular shape.

Szabo and coworkers have carried out several theoretical investigations on the nature and functional significance of a variety of dynamical processes in biological systems. He has developed a comprehensive analytical theory for the polarized photolysis experiment with intense light pulses which has been applied successfully to experimental results on the dynamics of myoglobin and hemoglobin. In collaboration with Chen and Rubin, Szabo has found an analytic solution to the problem describing fluorescence self-quenching in cell fusion experiments which greatly facilitates the analysis of the results. This theory is based on the realization that diffusive transfer of probes between two cell surfaces connected by a pore can be accurately described by two-state chemical kinetics. Szabo has also made important advances in improving the Rouse-Zimm theory for polymer dynamics.

Zwanzig's research during the past year has focussed on polymer and macromolecule dynamics. He has applied the general theory of hydrodynamic coupling to the interpretation of measurements of translational and rotational diffusion in order to obtain more accurate size and shape information from such data. Zwanzig is also investigating the general theory of the coupling of local and global motions of polymers in order to give a more accurate description of the dynamics of unfolded proteins. Robert Zwanzig was the recipient of the American Chemical Society Hildebrand Award for his seminal work on the theoretical chemistry of liquids.

Chen has continued to carry out theoretical studies on the bioenergetics and kinetics of free energy transducing systems. His analysis of data on the distance of sliding between actin and myosin filaments associated with the hydrolysis of a single ATP molecule are consistent with the conventional model for the mechanism of muscle contraction, in contradiction to recent claims. In another investigation he has found that the random fluctuations in the times of duration of positive and negative membrane potentials lead to the same frequency and amplitude dependencies of active transport of ions across red cell membranes as in the case where durations of the membrane potentials are regular.

McDiarmid is carrying out optical spectroscopic studies on the electronic structure and conformation of the excited states of small polyatomic molecules that are models for more complex biological chromophores. The results of studies on jet cooled samples of norbornadiene are being used to test various theoretical methods for calculating electronic structure. Investigations of acetone reveal a change in the dynamics of the excited Rydberg electronic state consisting of a change in the CCC angle deformation force constant.

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Levin, Lewis and coworkers are using Raman and infrared spectroscopy to investigate several different biological systems. They have also continued their development of a novel vibrational spectroscopic imaging system which combines the spatial resolving power of optical microscopy and the selectivity of infrared and Raman spectroscopy. By simultaneously measuring the resonance Raman spectra hemes  $a$  and  $a_3$  of cytochrome oxidase during a potentiometric titration these investigators have discovered have shown that the two hemes are not equally reduced at all voltages, arguing against the classical model for the redox behavior of this enzyme. Levin and colleagues have also continued their studies on the structural and dynamical properties of intact and model membranes, with emphases on the study of the effects of synthetic peptide based surfactants which are potential agents for the treatment of respiratory distress syndrome in newborn infants, and the investigation of the interactions of bile salts with membranes in which they have found a correlation between the hydrophobicity of the bile salt and the melting temperature of the membrane phase transition

Vibrational spectroscopic studies have been undertaken on model systems to clarify the factors governing the balance of forces leading to an interdigitated chain membrane bilayer morphology. Studies have also continued on the design of . From Raman spectroscopic temperature profiles the degree and mechanism by which peptides alter membrane disorder and dynamics can be determined. These techniques are also being used to investigate the interaction of bile salts with model membranes. The bile salt ursodeoxycholate is found to induce domains of interdigitated chain lipids within the membrane.

Eaton, Hofrichter and Henry are using time resolved optical spectroscopy in photodissociation experiments to investigate folding and structure function relations in heme proteins. Fast events in the folding of cytochrome  $c$  are being studied by optically triggering the folding reaction with nanosecond laser pulses. Before folding begins transient binding of both non-native and native ligands from the unfolded polypeptide are observed on a microsecond time scale. In the structure-function studies this group finds that all of the major features of myoglobin kinetics at ambient temperatures can be explained with a "minimal" model that includes a fast and slow rebinding conformation and two geminate states for each conformation. They also find that the rate of escape of the photodissociated ligand from the energy well adjacent to the heme calculated from molecular dynamics simulations is in good agreement with the value calculated from experimental data, suggesting that multiple geminate states should be an integral part of a kinetic model. Eaton and Hofrichter have begun to investigate the role of increased intracellular polymerization as a cause of more severe clinical severity in patients with homozygous sickle cell disease by using their laser photolysis - light scattering technique on individual cells to measure the distribution of intracellular delay times in patients with varying degrees of clinical severity and comparing these distributions before and after the beginning of hydroxyurea therapy.

Ziffer is synthesizing novel artemisinin derivatives that are potential agents for the treatment of malaria. Several hydroxy and carbonyl derivatives of 12 $\beta$ -alkyldeoxoartemisinin were converted to the corresponding fluorinated derivatives and found to be potent antimalarial compounds in *in vitro* tests. Several other derivatives have been prepared which are at various stages of evaluation in *in vitro* testing and mouse toxicity studies.



Hagins and Yoshikami are investigating the mechanism of visual transduction with calorimetric, X-ray microanalytic, and photometric methods and by studying images obtained after the introduction of fluorochromic dyes into retinas. They are also developing new techniques for introducing polar molecules into brain and retinal tissue based on substitution with silyl esters or ethers. The silyl derivatives penetrate the lipid membrane rapidly and are then slowly hydrolyzed by the cell water, trapping them inside the cell.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29001-21-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (60 characters or less. Title must fit on one line between the borders.)

Molecular dynamics and vibrational characteristics of membrane assemblies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Ira W. Levin Research Chemist LCP-NIDDK

Others: E. Neil Lewis Visiting Associate LCP-NIDDK

Tonya Herne IRTA LCP-NIDDK

Maria T.-Cotisel Special Volunteer NIAMS

Willis Person IPA LCP-NIDDK

COOPERATING UNITS (if any)

C. Huang, School of Medicine, Univ. of VA; J.S. Vincent, Univ. of MD; R. Hendler, NHLBI; C. G. Cochrane, Research Institute of Scripps Clinic, CA.; P. Harmon, Liposome Co., NJ.

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

4.5

PROFESSIONAL:

4.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A. Resonance Raman spectroscopy and optical spectroscopy were used to monitor the redox state of Heme A<sub>3</sub>, having a and a<sub>3</sub> centers, in cytochrome oxidase during potentiometric titrations. A novel approach was derived for obtaining simultaneous resonance Raman and optical absorption measurements of dilute protein solutions whose titrations curves are controlled potentiometrically. Quantitative Raman intensities, excited at 441.6 nm and measured as a function of solution voltage, yield the midpoint potential of the heme A<sub>3</sub> centers. The techniques were first tested by deriving the well-known midpoint potential of cytochrome c. Cyanide-inhibited cytochrome oxidase was examined as a simplified model of the native enzyme in which the heme a<sub>3</sub> is locked into the oxidized state. The spectroscopic analyses indicate that even when heme a<sub>3</sub> cannot undergo oxidoreduction, the heme a<sub>3</sub> centers show effective E<sub>m</sub>'s near 260 and 350 mV. Measurements on native (unbound) cytochrome oxidase give two effective E<sub>m</sub> values near 260 and 350 mV for heme a and lower E<sub>m</sub>'s near 260 and 350 mV for the heme a<sub>3</sub> centers. These results argue against the Neoclassical model of cytochrome redox behavior in which the heme a and heme a<sub>3</sub> centers are predicted to be equally reduced at all voltages. That is, these findings are consistent with recent reports of differential heme a and heme a<sub>3</sub> redox behavior.

B. A solid-state acousto-optic tunable filter (AOTF) was combined with krypton laser excitation, holographic filters and a photon-counting silicon avalanche photodiode detection to construct a miniaturized Raman spectrometer with no moving parts. The miniature AOTF spectrometer is used as an accessory to a Raman imaging microscope system in our laboratory. Although this spectrometer is optimized for the collection of Raman microspectra, it also functions as a microspectrometer for fluorescent studies.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29005-19-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Asymmetric Synthesis: Structure, Stereochemistry, and NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.'s Herman Ziffer Research Chemist LCP/NIDDK

Others: Yuming Pu Visiting Fellow LCP/NIDDK  
Boris Yagen Guest Worker LCP/NIDDK

COOPERATING UNITS (if any)

Dr. Sanford Markey, LCS/NIMH; Dr. H. H. C. Yeh, LAC/NIDDK

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.75

PROFESSIONAL:

2.75

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Artemisinin 1 has served as a lead compound in the development of new antimalarial drugs to treat resistant strains of *Plasmodium falciparum*. Malaria is the most widespread parasitic disease infecting some 270 million people worldwide and causing one to two million deaths per year. Many strains of *P. falciparum* from Southeast Asia have become resistant to the drugs currently used and threaten to spread to other areas of Asia, Africa and Central and South America. We first employed a fungus *Beauveria sulfurescens* to introduce hydroxy groups into  $\beta$ -arteether 2 in an effort to improve its therapeutic index. The hydroxylated compounds were used as intermediates in several syntheses. As a number of fluorinated steroids, prostaglandins, nucleosides, amines, etc. are valuable medicinals and since no fluorinated artemisinin derivatives were known, we used the hydroxy arteethers as intermediates to prepare fluorinated arteethers. Several artemisinin derivatives, containing carbonyl groups, were also transformed into geminal difluoro derivatives. All the fluorinated compounds were as active as arteether (which is scheduled for clinical testing) against resistant clones of *P. falciparum*.

To examine the influence of the existing stereochemistry on its antimalarial activity, we sought new methods to alter the stereochemistry of groups in 1. That was done by preparing anhydrodihydroartemisinin 3 from dihydroartemisinin 4 and examining its chemistry. Compound 3 was converted into the  $\beta$ -epoxide 5 with the KF-complex of m-chloroperbenzoic acid. The  $\beta$ -epoxide was converted into an  $\alpha,\beta$ -, 12 $\beta$ -dihydroxydihydroartemisinin, 6. Compound 3 was oxidized to 11 $\alpha$ , 12 $\alpha$ -dihydroxydihydroartemisinin 7 with osmium tetroxide. It was also employed as a starting material for the preparation of 11-[<sup>3</sup>H]-arteether 16. The reaction sequence used to prepare 16 was also used in the synthesis of a series of 11-epidihydroartemisinin ethers, 8. In the course of these studies two new rearrangements were discovered. The structure and stereochemistry of the rearrangement products were determined by 1D and 2D-NMR studies and mass spectrometry.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01-DK-29008-22-LCP

PERIOD COVERED

October 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Electric and molecular structure investigation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Ruth McDiarmid Research Chemist LCP-NIDDK

Others: Aharon Gedanken Special Volunteer LCP-NIDDK

Xing Xing Visiting Fellow LCP-NIDDK

COOPERATING UNITS (if any)

Leo Klasinc and Brank Kovac, Rudger Boscovic Institute, Zagreb, Croatia

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Spectroscopy and Structure

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.9

PROFESSIONAL:

1.9

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Optical absorption and 2 photon resonant multiphoton ionization (REMPI) and photoacoustic (PA) spectroscopic measurements on static samples and REMPI measurements on jet cooled samples of norbornadiene have been carried out to both interpret the spectrum of this molecule and to compare the results obtained by the different techniques. All three methods detected the structured 3s Rydberg  $\leftarrow$ X transition of norbornadiene but only the optical measurement could detect either of two diffuse valence transitions in this molecule. Comparisons of the REMPI and PA results indicated that the failure of these techniques to detect valence transitions is probably due to their small 2 photon cross sections. The 3s Rydberg  $\leftarrow$ X transition of norbornadiene was also analyzed.

Polarization-selected, 2- and 3-photon REMPI measurements were carried out on the 3p Rydberg  $\leftarrow$ X transitions of acetone. The results were interpreted to indicate a significant reduction in the CCC angle deformation force constant on excitation and a coupling of one of these Rydberg states to the  $\pi$ - $\pi^*$  excited state of acetone. From the latter, the  $\pi$ - $\pi^*$  excited state was estimated to lie approximately 8 eV above the ground state of the molecule.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01-DK-29010-21-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Dynamics of Proteins and Studies on Sickle Cell Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	William A. Eaton	Medical Officer	LCP-NIDDK
Others:	James Hofrichter	Research Chemist	LCP-NIDDK
	Eric R. Henry	Research Chemist	LCP-NIDDK
	Anjum Ansari	Visiting Associate	LCP-NIDDK
	Colleen M. Jones	Staff Fellow	LCP-NIDDK
	Garrott Christoph	Expert	LCP-NIDDK

COOPERATING UNITS (if any)

Andrea Mozzarelli, Institute of Biochemical Sciences, University of Parma, Italy

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Time resolved optical spectroscopy in photodissociation experiments and molecular dynamics simulations are being used to investigate protein folding and structure function relations in heme proteins. Fast events in the folding of cytochrome c are being studied by optically triggering the folding reaction with nanosecond laser pulses. Before folding begins we observe transient binding of both non-native and native ligands from the unfolded polypeptide on a microsecond time scale. This optical trigger should provide a powerful method for studying chain collapse and secondary structure formation in cytochrome c without any limitations in time-resolution.

Kinetic and theoretical studies on ligand binding in myoglobin have yielded new important information on the mechanism, including the finding that the viscosity dependence of the protein conformational relaxation rate can be explained using a modification of Kramers theory which includes the contributions of both protein and solvent to the friction, the demonstration that all of the major features of myoglobin kinetics at ambient temperatures can be explained with a "minimal" model that includes a fast and slow rebinding conformation and two geminate states for each conformation, and the finding that the rate of escape of the photodissociated ligand from the energy well adjacent to the heme calculated from molecular dynamics simulations is in good agreement with the value calculated from experimental data, suggesting that multiple geminate states should be an integral part of a kinetic model.

We have begun to investigate the role of increased intracellular polymerization as a cause of more severe clinical severity in patients with homozygous sickle cell disease by using our laser photolysis - light scattering technique on individual cells to measure the distribution of intracellular delay times in patients with varying degrees of clinical severity and comparing these distributions before and after the beginning of hydroxyurea therapy.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29011-22-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The physics and chemistry of photoreception

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.	William A. Hagins	Medical Officer	LCP-NIDDK
Others:	S. Yoshikami	Research Biologists	LCP-NIDDK
	P. Ross	Research Chemist	LMP-NIDDK
	K. Spring	Research Med. Officer	LKM-NHI
	Mark Vivino	Computer Systems Analyst	CSL-DCRT
	L. Pannell	Research Chemist	LAC-NIDDK

COOPERATING UNITS (if any)

Sudhir Sahu and Sheila Shah, Student Volunteers, Thomas Jefferson High School, Alexandria, VA, Ramin Rashidian, Student Volunteer, Murray State University, Murray, KY 42071.

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Membrane Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER

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- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Calcium metabolism in retinal rod outer segments during phototransduction is being studied with the aid of fluorochromic dyes introduced into retinas by new methods and studied by quantitative microscopic image systems.

Calorimetric, X-ray microanalytic and photometric methods are being used to study ionic and biochemical events during phototransduction.

New techniques for breaching the blood-brain barrier to introduce polar molecules into brain and retinal tissue are under study.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29016-16-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Macromolecular dynamics and assembly reactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: James Hofrichter Research Chemist LCP-NIDDK

Others: Garrett Christoph Special Expert LCP-NIDDK  
William A. Eaton Medical Officer LCP-NIDDK  
Chi-Kin Chan Visiting Associate LCP-NIDDK  
Yi Hu Visiting Associate LCP-NIDDK  
Eric Henry Research Chemist LCP-NIDDK  
Colleen Jones Visiting Fellow LCP-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Laser Biophysics and Spectroscopy

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues X (c) Neither  
(a1) Minors  
(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

**Time-resolved absorption spectroscopy** is used to study the **dynamics of protein structural changes** subsequent to excitation with short laser pulses. Molecular models for the protein dynamics are used to fit and interpret the measured data.

- A. The kinetics of ligand binding and conformational changes for **sperm whale myoglobin** and **human hemoglobin** (HbA) have been studied following the **photodissociation of carbon monoxide** from the hemes. Comprehensive sets of data from partial photolysis experiments using polarized excitation permit cooperative and noncooperative processes to be clearly distinguished. These data also provide accurate measurements of the rotational correlation times of these molecules
- B. The dynamics of short-lived states on the folding pathway of **cytochrome c** have been investigated using time-resolved absorption spectroscopy. The binding of carbon monoxide destabilized the folded state of reduced cytochrome c, permitting folding to be initiated by photodissociation of this ligand. We have observed transient binding of other at least two different side chains to the heme subsequent to photodissociation of CO.
- C. We have disassembled, moved, rebuilt and improved all of the instrumentation used by our laboratory.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01-DK-29017-13-LCP

PERIOD COVERED

October 1, 1993 through September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Spectroscopic investigation of membrane lipids and models

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Ralph G. Adams Research Physicist LCP-NIDDK

Others: Ira W. Levin Research Physicist LCP-NIDDK

COOPERATING UNITS (if any)

Sherwin Strauss (FDA)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Molecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

0

PROFESSIONAL:

0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Project has been terminated.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01-DK-29019-13-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Theoretical studies on the dynamical aspects of macromolecular function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. A. Szabo Research Chemist LCP-NIDDK

Others: X. Zhou Visiting Associate LCP-NIDDK

A. Ansari Visiting Associate LCP-NIDDK

COOPERATING UNITS (if any)

Chen Y., and Rubin R., - (all) LCP-NIDDK; R. Pastor, FDA; Freed K., Chicago; McCannon A., Houston; Perico A., Genova; Maroncelli M., University Park; Bagchi B., Bangalore.

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Theoretical Biophysical Chemistry Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

3.0

OTHER

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The theory of absorbance measurements on a system (e.g. a chromophore in a protein) that undergoes a sequence of reactions initiated by a linearly polarized laser pulse of arbitrary intensity is developed. In order to provide a complete framework that could be used to analyze experiments, a wide variety of complications (e.g. reorientational motion on the same time scale as the intense excitation pulse, the influence of internal motions and chemical kinetics) were explicitly treated. In order to describe the dynamics of macromolecules on time scales where conformational transitions (barrier crossings) occur, we showed that memory function corrections must be added to the optimized Rouse-Zimm theory and developed a new, computationally efficient way of doing so. A simple analytic theory required to analyze fluorescence studies of cell-cell fusion has been developed. This theory is based on the realization that the diffusive transfer of probes between two cell surfaces connected by a small pore can be accurately described by two state chemical kinetics. The rate constants were expressed in terms of microscopic parameters by exploiting a rigorous mapping of the cell problem onto one involving diffusion on a one dimensional bistable potential with an entropic barrier. An analytic theory for the response of a dipolar lattice to a newly created change has been formulated based on a dynamic mean spherical approximation. The predictions of this theory for solvation dynamics has been tested against computer simulations. The influence of electrostatic interactions and diffusion on the rate of protein-protein association kinetics have been examined. A new method (boundary elements) for calculating the electrostatic interaction energy between two macromolecules has been developed. This procedure can handle the irregular shape of the molecular surfaces, the small dielectric constant in the interior and the presence of salt ions in solution.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29020-08-LCP

PERIOD COVERED

October 1, 1993 through September 30, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nuclear magnetic resonance: new methods and molecular structure determination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Ad Bax Visiting Scientist LCP-NIDDK

Others: Rolf Tschudin, Electronics Engineer, Frank Delaglio, Special Expert  
Jacob Anglister, Visiting Scientist, Andy Wang, IRTA, Geerten Vuister, Visiting  
Fellow, Stephan Grzesiek, Visiting Associate, David Live, Special Volunteer  
Guang Zhu, IRTA, James Ernst, Student Volunteer - all LCP/NIDDK.

COOPERATING UNITS (if any)

Marius Clore, Angela Gronenborn, LCP-NIDDK; Dennis Torchia, LBR-NIDR; Claude Klee,  
LC-NCI

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Biophysical Nuclear Magnetic Resonance Spectroscopy Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

7.25

PROFESSIONAL:

7.25

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

A new NMR approach has been developed for the measurement of three-bond J couplings. The approach is applicable to isotopically enriched macromolecules and to concentrated solutions of molecules at natural abundance. The three-bond J couplings between atomic nuclei provide very direct information on the magnitude of the intervening dihedral angles and thereby yield valuable new constraints in the structure determination process of biological macromolecules. The approach has been successfully applied to the measurement of  $^{13}\text{C}$ - $^{13}\text{C}$ ,  $^{13}\text{C}$ - $^{15}\text{N}$ ,  $^1\text{H}$ - $^{15}\text{N}$ ,  $^1\text{H}$ - $^{13}\text{C}$ ,  $^1\text{H}$ - $^{113}\text{Cd}$ , and  $\text{H}^{\text{N}}\text{-H}^{\text{H}}$  J couplings in a range of proteins.

It has been demonstrated for the first time that a detailed structural analysis of a detergent-solubilized protein is possible without recourse to perdeuterated detergent. This type of analysis relies on the combined use of  $^{13}\text{C}$ - and  $^{15}\text{N}$ -edited NMR experiments and pulsed field gradients which make it possible to suppress the intense detergent  $^1\text{H}$  NMR signals while observing the much weaker protein signals. The approach is demonstrated for the protein calcineurin B, solubilized by a 15 molar excess of the detergent CHAPS. The calcineurin A/B complex remains fully active in the presence of CHAPS and NMR results confirm that the detergent does not significantly alter the protein structure. Resonance assignment of the backbone  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$  resonances and the secondary structure determination process have been completed. It is evident that the secondary structure of calcineurin B is highly homologous with that of the smaller protein calmodulin.

10.1 10.2 10.3 10.4 10.5 10.6 10.7 10.8 10.9 10.10 10.11 10.12 10.13 10.14 10.15 10.16 10.17 10.18 10.19 10.20 10.21 10.22 10.23 10.24 10.25 10.26 10.27 10.28 10.29 10.30 10.31 10.32 10.33 10.34 10.35 10.36 10.37 10.38 10.39 10.40 10.41 10.42 10.43 10.44 10.45 10.46 10.47 10.48 10.49 10.50 10.51 10.52 10.53 10.54 10.55 10.56 10.57 10.58 10.59 10.60 10.61 10.62 10.63 10.64 10.65 10.66 10.67 10.68 10.69 10.70 10.71 10.72 10.73 10.74 10.75 10.76 10.77 10.78 10.79 10.80 10.81 10.82 10.83 10.84 10.85 10.86 10.87 10.88 10.89 10.90 10.91 10.92 10.93 10.94 10.95 10.96 10.97 10.98 10.99 11.00



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01-DK-29021-08-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Conformation and dynamics of biological molecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Eric Henry Research Physicist LCP-NIDDK

Others: William Eaton Medical Officer LCP-NIDDK  
 Colleen Jones Staff Fellow LCP-NIDDK  
 Olivier Schaad Visiting Associate LCP-NIDDK  
 Attila Szabo Research Chemist LCP-NIDDK  
 Huan-Xiang Zhou Visiting Associate LCP-NIDDK  
 Chi-Kin Chan Visiting Associate LCP-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Biophysical Chemistry Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

We have used time-resolved optical spectroscopy to study the refolding of reduced cytochrome c in guanidine hydrochloride solutions initiated by the photodissociation of bound CO. Several spectral changes are observed at times ranging from microseconds to tens of milliseconds, which are attributed to both CO rebinding and the transient binding of non-native heme ligands from among the amino acid sidechains of the unfolded protein. We have analyzed the measured spectra using a simple kinetic model in which the two histidine and two methionine sidechains of the protein may compete with CO to bind to the heme. Fits with this model are consistent with the methionines binding more rapidly than the histidines, with however very little transient formation of folded protein with the native methionine 80 bound. We have also simulated the photodissociation and subsequent rebinding of nitric oxide ligands to myoglobin using molecular dynamics. Most of the dissociated ligands remain within a pocket close to the heme before rebinding within a few picoseconds. The simulated rate of rebinding at the heme is considerably faster than the experimentally determined rate, but the rate of ligand escape from the heme pocket estimated from the simulations using a simple kinetic scheme is comparable to the corresponding estimate from the experiments.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29022-06

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural Studies of AIDS proteins by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.'S	Angela Gronenborn	Visiting Scientist	LCP-NIDDK
	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Ad Bax	Visiting Scientist	LCP-NIDDK
Others	Daniel Garrett	IRTA Fellow	LCP-NIDDK
	Patricia Lodi	Special Volunteer	LCP-NIDDK
	Jim Omichinski	Staff Fellow	LCP-NIDDK
	Robert Powers	IRTA Fellow	LCP-NIDDK

COOPERATING UNITS (if any)

NCI (Ettore Appella, K. Sakaguchi); Immunex (Carl March); Protein Expression Laboratory (Stephen Stahl, Paul Wingfield); LMB-NIDDK (Gary Felsenfeld and C. Trainor); University of Newcastle, U.K. (Roger Pain); NHLBI (Warren Leonard).

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Sections on Structural Biology, Protein NMR, and Biophysical NMR Spectroscopy

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Work has been carried out on a number of structural problems related to proteins derived from the HIV virus. These include the p7 nucleocapsid protein of HIV-1, and proteins of the immune system, in particular interleukin-1 $\beta$ , interleukin-4 and the double zinc finger of the human enhancer binding protein MBP-1. The solution structure of interleukin-4 has now been refined to very high resolution. The RNA binding properties of the p7 nucleocapsid protein have been investigated as a prelude to structural studies of a specific p7-RNA complex. The kinetics and pathway of folding of interleukin-1 $\beta$  has been investigated using a combination of NMR, CD and fluorescence. Finally, the solution structure of the specific complex of the DNA binding domain of the erythroid transcription factor GATA-1 with its DNA target site has been determined. The latter is of interest as the highly homologous DNA binding domain of the lymphoid transcription factor GATA-3 interacts with a GATA sequence within the LTR of the HIV-1 genome, stimulating transcription by a factor of 6-8.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29023-03-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Determination of Three-Dimensional Structures of Macromolecules in Solution by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK
Other:	Daniel Garrett	IRTA Fellow	LCP-NIDDK
	Bruce Grasberger	IRTA Fellow	LCP-NIDDK
	John Kuszewski	Special Volunteer	LCP-NIDDK
	Patricia Lodi	Guest Researcher	LCP-NIDDK
	Jim Omichinski	Staff Fellow	LCP-NIDDK
	Robert Powers	IRTA Fellow	LCP-NIDDK

COOPERATING UNITS (if any)

Laboratory of Chemical Physics, NIDDK (Ad Bax) Laboratory of Molecular Biology, NIDDK (Gary Felsenfeld), Protein Expression Laboratory (Stephen Stahl, Paul Wingfield); NCI (Ettore Appella); NHLBI (Warren Leonard); Yale (Axel Brünger, Ken

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Sections on Protein NMR and Structural Biology

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Work in this laboratory has been focussed on the determination of three-dimensional structures of larger proteins in solution by NMR, with a particular emphasis on cytokines, immune related proteins and DNA-protein complexes. A considerable effort has been placed on the development of three- and four-dimensional heteronuclear NMR to extend the application of NMR as a method for determining three-dimensional structures of proteins in solution beyond the limits of conventional two-dimensional NMR (~100 residues) to molecules in the 150- to 400-residue range. In addition, studies have been carried out to assess the limits of precision and accuracy that can be attained in an NMR structure determination and to assess the quality of the structures by complete cross-validation.

High resolution solution structures of a number proteins have been determined. These include the cytokine interleukin-4 and the complex of the DNA binding domain of the erythroid transcription factor GATA-1 with its specific DNA target site. Extensive use in these studies has been made of multi-dimensional heteronuclear NMR and of systematic conformational searches to obtain stereospecific assignments and torsion angle restraints which have enabled us to obtain very high resolution structures comparable in accuracy to 2 Å resolution X-ray structures. The typical accuracy attainable is 0.2-0.4 Å for the backbone atoms and 0.4-0.5 Å for the internal side chains.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29025-04-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Investigations of Macromolecular Structures and Dynamics in Solution by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.'S	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK
	G. Marius Clore	Visiting Scientist	LCP-NIDDK
Others:	Daniel Garrett	IRTA Fellow	LCP-NIDDK
	Bruce Grassberger	IRTA Fellow	LCP-NIDDK
	John Kuszewski	Special Volunteer	LCP-NIDDK
	Patricia Lodi	Guest Researcher	LCP-NIDDK
	Jim Omichinski	Staff Fellow	LCP-NIDDK
	Robert Powers	IRTA Fellow	LCP-NIDDK

COOPERATING UNITS (if any)

Laboratory of Chemical Physics, NIDDK (Ad Bax) Laboratory of Molecular Biology, NIDDK (Gary Felsenfeld), Protein Expression Laboratory (Stephen Stahl, Paul Wingfield); NCI (Ettore Appella); NHLBI (Warren Leonard); Yale (Axel Brünger, Ken

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Sections on Structural Biology and Protein Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unaducted type. Do not exceed the space provided.)

The objective of the overall research in this laboratory is centered on achieving as complete a description as possible for the structures of peptides, proteins, nucleic acids and their complexes in solution, principally by NMR spectroscopy. At present particular emphasis is being placed on developing approaches which allow the investigation of larger and complex systems as well as increase the precision with which these solution structures can be obtained, studies aimed at correlating structure and function, and experiments aimed at investigating protein folding.

Structures for several proteins have been determined and analyzed. These include the cytokine interleukin-4, and the complex of the DNA binding domain of the erythroid transcription factor GATA-1 with its specific DNA target site. These studies have exploited many novel 3D and 4D heteronuclear NMR experiments to dramatically increase spectral resolution and thereby resolve assignment ambiguities in larger proteins. The dynamics of the cytokine interleukin-8 have been explored. The contact surface of the IgG binding domain of Streptococcal G complexed with a human IgGfc has been identified. Finally, the folding pathway and kinetics of the all  $\beta$ -sheet protein interleukin-1 $\beta$  have been investigated.

$\frac{d}{dt} \left( \frac{\partial L}{\partial \dot{x}} \right) = \frac{\partial L}{\partial x}$



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29026-05-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

NMR and other spectroscopic studies of molecular structure

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Edwin D. Becker Research Chemist LCP-NIDDK

Others: Drazen Vikić-Topic Visiting Associate LAC-NIDDK

COOPERATING UNITS (if any)

Molecular Graphics and Simulation Laboratory, DCRT  
Sophisticated Instruments Facility, Indian Institute of Science, Bangalore, India  
Chemistry Department, Rudjer Boskovic Institute, Zagreb, Croatia

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.7

PROFESSIONAL:

1.7

OTHER:

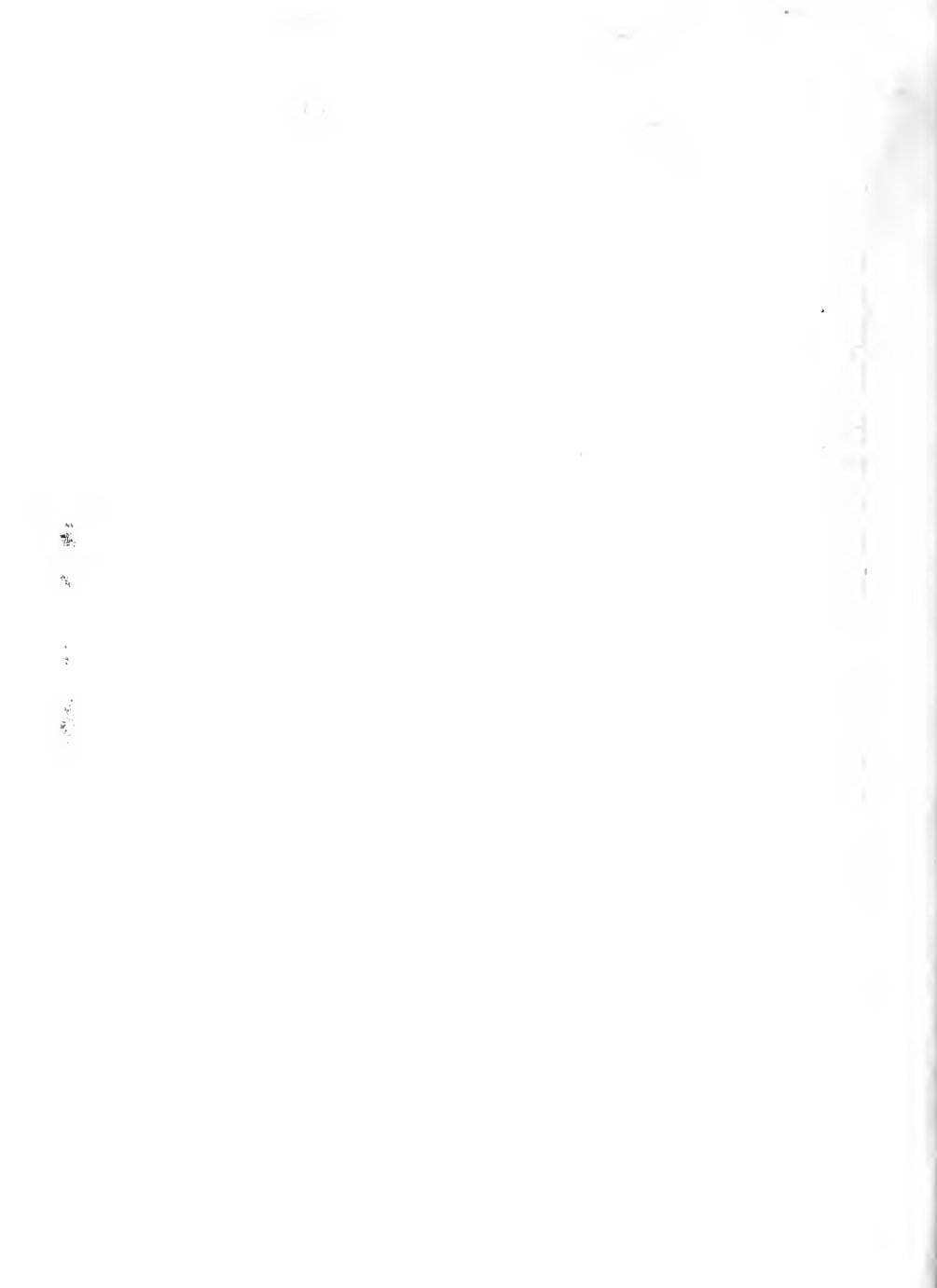
CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are exploring the mechanism and potential applications of the effect of isotopic substitution on NMR chemical shifts of nuclei that are several bonds away from the site of substitution. Studies of a number of differently substituted binuclear aromatic molecules (including *cis*- and *trans*-stilbenes, *trans*-azobenzene, diphenylacetylene and diphenylethane) show isotope-induced changes in carbon-13 chemical shifts through as many as 10 chemical bonds and as small as 0.64 parts per billion. *Ab initio* molecular orbital calculations for *trans*-stilbene demonstrate that a small change in the average length of the C-H/C-D bond (simulated by a decrease of 0.012 Angstrom unit on deuteration) causes changes in the computed carbon-13 chemical shifts that agree very well with experimental observations.

Studies with liquid crystal solvents are being used to study weak molecular interactions. This method permits the measurement of very small distortions in molecular shape. Although extremely small distortions of other highly symmetric molecules (such as methane) are readily observed, fullerene (C-60) has been found to retain its spherical symmetry.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01-DK-29027-05-LCP

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Theoretical studies of dynamical processes in chemical physics and biophysics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Robert Zwanzig

Research Chemist

LCP-NIDDK

Others:

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Theoretical Biophysics Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Several different topics were studied. (1) A general survey was made of experiments and theory of bacterial chemotaxis. The main interest was to see if one could establish a connection between the essentially biophysical measurements of Berg and others, and the essentially biochemical investigations of Koshland and others. This work continues. (2) When an unsymmetrical molecule (e.g. a protein) moves in a viscous liquid, there is a coupling of viscous forces and torques due to its translation and rotation. When this coupling occurs, it can seriously affect the interpretation of measured translational and rotational diffusion coefficients in terms of molecular size and shape. The theory of this hydrodynamic coupling due to Wegener was verified and then worked out explicitly for a deformed sphere. (3) Two kinds of motion can occur in polymers; one is a local motion involving, e.g., conformational changes, and the other is a global or librational motion involving the entire polymer. Moroz pointed out that there can be interactions between these two kinds of motion. This requires a re-working of some of the theory of polymer solution dynamics.

1882

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29028-03-LCP

PERIOD COVERED

October 1992 through September 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Free energy conversion in biology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Yi-der Chen Research Chemist LCP-NIDDK

Others: Robert J. Rubin Special Volunteer LCP-NIDDK

COOPERATING UNITS (if any)

B. Brenner, Dept of Physiology, University of Ulm, Ulm, Germany  
T. Y. Tsong, Dept. of Biochemistry, The Hong Kong University of Science and Technology, Hong Kong.

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Theoretical Biophysics Section

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The general aim of this project is to study the molecular mechanisms of free energy transduction in biology, such as in muscle contraction, active transport, etc.. During this period, a number of different topics related to this project have been studied. The most important areas in which progress has been made are: (1) A theoretical model simulation study on the response of contracting muscles to multiple periodic perturbations in force and length. The results of the calculations are used to resolve the question of how many power-strokes (or rowing motions) an actomyosin crossbridge in a contracting muscle fiber can perform during one ATP hydrolysis cycle; (2) A theoretical model study of experimentally measured active transport of Rb+ ions across red blood cell membranes induced by a randomly fluctuating electric field. The parameters of a four-state carrier model that fits experimental data are obtained; (3) The study of diffusion and dequenching of self-quenching fluorophores in membranes of two fused cells. A simplified formula for the dequenching kinetics is obtained, which is useful in elucidating the mechanisms of the glycoprotein-mediated cell-cell fusion reaction.



## NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research of the Laboratory is directed towards the introduction of new concepts, techniques and agents for the elucidation of the molecular nature of mechanisms controlling cell functions. Specific focus is placed on i) Development of selective agonists/antagonists for receptors controlling cyclic nucleotide formation, phospholipid metabolism and ion channel function; ii) The relationship between ion transport, phospholipid turnover and cyclic nucleotide generation and the delineation of agents with specific effects on macromolecules involved in these systems. iii) The isolation and structure elucidation of biologically active natural products and definition of the basis of their activity. iv) Effects of agents on ion channels and the development of radioactive ligands for modulatory sites in such channels. v) The nature of enzymes involved in formation and inactivation of neurotransmitters, hormones, and other modulatory substances, in particular the enzymes, catechol-O-methyltransferase, monoamine oxidase, adenylate cyclase and phosphodiesterases. vi) the fundamental mechanisms by which drugs and environmental chemicals are transformed in the body with emphasis on oxidative metabolism by cytochrome P-450 systems to generate active oxide metabolites that interact with macromolecules such as DNA and are metabolized by further oxidation, by hydrolysis and by conjugation with glutathione.

Some of the milestones for the Laboratory are as follows: i) Introduction of the adenine-prelabeling technique for study of cyclic AMP generation in intact cells; ii) The steroidal alkaloid batrachotoxin as a selective activator of sodium channels. iii) Histronicotoxin as a noncompetitive blocker of acetylcholine receptor channels and potassium channels. iv) Pumiliotoxins as myotonic and cardiotoxic alkaloids acting through sodium channels to elicit phosphoinositide turnover. v) N<sup>6</sup>-Substituted adenosines and 8-phenyl and 8-cyclohexylxanthines and other heterocycles as selective and potent adenosine receptor agonists and antagonists suitable as radioligands for binding studies and for definition of A<sub>1</sub> and A<sub>2</sub> classes of receptors. vi) Introduction of forskolin as a specific and widely useful activator of adenylate cyclase. vii) Fluoronorepinephrines and analogs as selective alpha and beta-adrenergic agonists. viii) Production of antibodies to catechol-O-methyl transferase and their use in studying localization of this key catechol-metabolizing enzyme. ix) Discovery of a potent non-opioid analgetic alkaloid, epibatidine. x) Definition of a relationship between receptor-activation of phosphoinositide breakdown; protein kinase C activation, and altered responses of cyclic AMP-generating systems. xi) Introduction of maitoxin as a general activator for phosphoinositide breakdown and receptor-operated calcium channels. xii) Discovery of the NIH shift of aryl substituents during P-450 catalyzed phenol formation and demonstration of arene oxides as intermediates. xiii) Demonstration of oxidation-hydrolysis pathways that convert stereoselectively polycyclic aromatic hydrocarbons to ultimate diol epoxides that react selectively with DNA. xiv) Discovery and formulation of the bay-region theory, which is predictive of the pathway for formation of reactive carcinogenic metabolites from polycyclic aromatic hydrocarbons. xv) Development of optical assays for protease and reverse transcriptase of HIV-1.

The laboratory accomplishes its mission both through its own resources and





through extensive collaborations with other laboratories both at NIH, at Universities, Museums, and other institutes and in drug and chemical companies. Such collaborations can involve sharing of expertise on syntheses, isolations, analyses and biological testing and field work to obtain sources of new natural products.

## SECTION ON PHARMACODYNAMICS

### Pharmacologically Active Compounds from Amphibians and Other Natural Sources

Structure elucidation of alkaloids from skin of frogs and toads continues to reveal unique new compounds many with high biological activity. Syntheses for several are under investigation or have been completed. These include the myotonic homopumiliotoxins, the pseudophrynamines that, based on structural analogy to physostigmine, may be acetylcholine esterase inhibitors, epibatidine, a novel pyridyl-nortropane with potent non-opioid analgetic activity, the pyrrolidine oximes and a proposed class of dehydro-homopumiliotoxins.

Alkaloids in Birds. The first example of toxicity and chemical defense in birds was discovered. Birds of the New Guinean genus Pitohui possess the steroidal alkaloid homobatrachotoxin, a toxin previously considered unique to neotropical poison-dart frogs of the dendrobatid genus Phyllobates. The birds also contain an alkaloid (M.W. 237,  $C_{12}H_{19}N_3O_2$ ) in muscle with local anesthetic activity. A vine, whose toxic berries are purportedly eaten by Pitohui birds, contains an unknown toxic alkaloid unrelated to the homobatrachotoxin. A Mexican bird, considered a food taboo by the Aztecs, contains an alkaloid with potent behavioral stimulant activity.

### Alkaloid Profiles in Amphibians: Genetic or Environmental Determinants.

Dendrobatid frogs produce a diverse set of skin alkaloids, whose profiles appear characteristic of frogs of each species or, in the case of variable species, of each population. Many such alkaloids also have been detected in Australian myobatrachid frogs of the genus Pseudophryne (pumiliotoxins, pyrrolizidine oximes and the unique pseudophrynamines), in the South American bufonid toads of the genus Melanophryniscus (pumiliotoxins, homopumiliotoxins, quinolizidines, indolizidines, pyrrolizidines, decahydroquinolines, pyrrolizidine oximes and coccinellines) and in the Madagascan mantellid frogs of the genus Mantella (pumiliotoxins, quinolizidines, pyrrolizidines, decahydroquinolines and unique alkaloids proposed to be dehydropumiliotoxins). Dendrobatid frogs raised in terraria do not contain skin alkaloids. It has now been demonstrated that captive-raised frogs raised on leaf-litter insects do contain quinolizidines, coccinellines and pyrrolizidine oximes commensurate with levels in wild-caught frogs, thereby establishing a dietary source for at least three classes of amphibian alkaloids. The leaf-litter insects that serve as the dietary source of these and potentially other "amphibian alkaloids" are being investigated. Dendrobatid frogs of the genera that normally contain skin alkaloids have very efficient systems for transport and accumulation of dietary alkaloids into skin. This process can be very selective, since the pyrrolidines and piperidines from ants are not accumulated, while ant indolizidines are. Non-alkaloid-containing dendrobatid frogs of the genus Colostethus do not have such alkaloid transport systems. One species of Colostethus does contain tetrodotoxin in its skin. The structures of further alkaloids from frog skin are under investigation.

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Syntheses of Amphibian Alkaloids. A pyrrolizidine oxime 236 has been synthesized. Preliminary pharmacological evaluation indicate that it may be a bombesin antagonist. Epibatidine has been synthesized. Preliminary in vivo studies indicate that both enantiomers have similar biological activity. Pseudophrynamine A has been synthesized, but further synthetic work is needed in order to obtain adequate supplies for biological evaluation as a cholinesterase inhibitor. Syntheses directed towards the myotonic homopumiliotoxins and the proposed dehydropumiliotoxins are in progress.

## SECTION ON PHARMACODYNAMICS

### Pharmacology and Metabolism of Biogenic Amines and Related Compounds

#### Catechol-O-methyltransferase(COMT):

The study of COMT activity in hamster kidney following estrogen-induced renal carcinogenesis is still in progress. Initial studies indicate the major site of COMT in the hamster kidney is in the epithelial cells of the proximal tubules with lesser amounts in the epithelial cells of the collecting ducts. Preliminary results suggests that the low levels of COMT found in hamster kidney is related to the sensitivity of hamster kidney to induction of renal carcinoma by estrogen. The mechanism of this sensitivity maybe related to the accumulation of 4-hydroxyestradiol and its participation in a redox cycle with the production of active oxygen species. The localization and level of COMT in estrogen receptor-positive and -negative endometrial and breast adenocarcinoma is still under study. Macrophages in cervical lymph nodes were shown to be a major site of both the extraneuronal uptake of norepinephrine and COMT.

## SECTION ON PHARMACODYNAMICS

### Ion Channels, Receptors and Second Messengers in the Nervous System

Maitotoxin: Site of Action. Maitotoxin appears to stimulate calcium influx via a so-called receptor-operated calcium (ROC) channel, thereby causing stimulation of phosphoinositide breakdown. Thus, the influx of calcium elicited by maitotoxin is inhibited in parallel with inhibition of phosphoinositide breakdown in cultured cells by imidazole compounds, such as SK & F 96365, proposed as inhibitors of ROC channels. A variety of imidazole compounds do inhibit MTX-elicited calcium flux in fibroblast cells, as do certain other calcium channel blockers. The most potent ( $IC_{50}$  1  $\mu$ M or less) blockers for the MTX-response are the imidazole L651582 (CAI), R-verapamil and dichlorobenzamil, the last an amiloride analog. Blockade by amiloride analogs requires for potency a benzyl substituent on the amidine function as in dichlorobenzamil and benzamil. Selective blockers of L-type, N-type and P-type voltage-dependent calcium channels are relatively ineffective versus maitotoxin-elicited calcium influx. The antimitotic activity, assayed versus thymidine incorporation in serum-stimulated fibroblasts, for the imidazoles and other agents does not correlate with activity versus maitotoxin-elicited calcium flux in fibroblasts.

ATP and Calcium Influx in Pheochromocytoma Cells: ATP and various analogues stimulate calcium influx in pheochromocytoma cells. The profile of activity, however, suggests that the receptor involved is of the  $P_{2y}$ -class coupled to



phospholipase C via a G-protein. ATP does stimulate phosphoinositide breakdown in PC12 cells and elicits a transient peak elevation of intracellular calcium followed by a sustained lower level of intracellular calcium. The transient elevation seems due to  $IP_3$ -triggered release of calcium, while the latter appears due to influx of calcium through ROC channels. In contrast, UTP although stimulating calcium influx, phosphoinositide breakdown and a transient elevation of intracellular calcium, does not caused a sustained elevation of calcium. ATP also causes presumably secondary to elevation of calcium, a release of catecholamines and an activation of calcium-dependent potassium channels. ATP elicited a greater influx of calcium in the absence of sodium, suggestive of a secondary activation of  $Na^+/Ca^{++}$  transporter. UTP causes none of these secondary events. A prior treatment with UTP causes a partial desensitization of ATP responses. The results suggest the presence of at least two purinergic receptors, one activated by ATP and leading to various sequelae including activation of ROC channels, the other activated by either ATP and UTP and causing only a transient influx of calcium and activation of polyphosphoinositide breakdown.

## SECTION ON OXIDATION MECHANISMS

### Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites

Studies have continued on the synthesis and chemistry of arene oxides. In an attempt to synthesize pure enantiomers of benz[a]anthracene 3,4-oxide from optically pure dibromo ester precursors, only racemic product was obtained. This racemization presumably occurred via an achiral oxepin, which was identified as a product of the synthetic reaction. Photoisomerization of benz[a]anthracene 3,4-oxide, benz[a]anthracene 1,2-oxide and triphenylene 1,2-oxide to the corresponding oxepins proceeds via an oxygen-walk process followed by electrocyclic ring expansion. Because of facile arene oxide-oxepin interconversion, such oxepins may play a role in the metabolism and mutagenicity of the corresponding arene oxides.

Rates and products of reactions of sixteen K-region arene oxides were investigated in methanol under acidic and basic conditions. A mechanism was proposed for the acid catalyzed reaction in which products are determined by partitioning of a hydroxy carbocation intermediate between solvent addition (to give cis and trans methanol adducts) and a conformational change which is required for the hydride migration (to give phenols). Rate constants for nucleophilic attack of methoxide ion at each position of unsymmetrical arene oxides were compared with the corresponding rate constants for acid catalyzed carbocation formation. These rate data were consistent with a transition state for the nucleophilic addition in which bond breaking of the epoxide is further advanced than bond making to the incoming methoxide ion. In their reaction with methoxide ion, nonplanar arene oxides which have severe steric crowding in the bay region exhibited a steric acceleration (ca. 0.9 kcal/mol) relative to planar arene oxides.

In a non-nucleophilic solvent, acetonitrile, K-region arene oxides rearrange to phenols via two sequential acid-catalyzed reactions: rapid rearrangement to the keto tautomers of the K-region phenols, followed by their enolization. Accumulation of the ketones allowed their characterization for the first time under solvolytic conditions. A linear correlation (slope 1.07) was observed



between the logarithms of the second-order rate constants for ketone formation in acetonitrile and those for the corresponding acid-catalyzed reactions of the arene oxides in methanol (where ketone does not accumulate). Negative deviations from this relationship were observed for the formation of ketones in which the carbonyl oxygen is *peri* to a methyl group. A mechanism was proposed in which the rate determining step for arene oxides that follow the correlation is oxirane ring opening in both acetonitrile and methanol, but changes from ring opening (in methanol) to conformational inversion of the hydroxy carbocation intermediate (in acetonitrile) for the *peri*-methyl compounds, as a result of steric interactions between the *peri*-methyl and hydroxyl groups. Once formed, the ketone intermediate enolizes relatively slowly in acetonitrile, presumably because the low basicity of this solvent retards proton abstraction from the *O*-methylene group of the *O*-protonated ketone. A linear relationship is observed between the Hückel  $\pi$ -bond character of the K-region bond in the parent hydrocarbon and the logarithm of the rate constant for acid-catalyzed enolization, consistent with the interpretation that development of aromaticity is a major driving force for enolization.

Benzo[e]pyrene-4,5-oxide is a meso K-region arene oxide which upon ring opening gives enantiomeric *trans*-4,5-dihydroxy-4,5-dihydrobenzo[e]pyrenes, whose absolute configurations were determined by an exciton chirality method. Rat liver microsomal epoxide hydrolase catalyzed the enantioselective hydration of the three meso arene oxides, benzo[e]pyrene 4,5-oxide, pyrene 4,5-oxide and phenanthrene 9,10-oxide, to give 83, 86, and 42% of the *trans*-(*R,R*)-dihydrodiol enantiomer, respectively.

A continuing focus of efforts in the Section has been in the characterization of covalent adducts between DNA and the carcinogenic diol epoxide metabolites of polycyclic aromatic hydrocarbons, at both the nucleoside and oligonucleotide levels. The principal covalent adducts formed from DNA upon reaction *in vitro* with the four optically active 3,4-diol 1,3-epoxides of benz[a]anthracene have been identified and their structures elucidated at the nucleoside level. These adducts include products of *cis* and *trans* addition to the epoxide of the exocyclic amino groups of deoxyadenosine (dA) and deoxyguanosine (dG) as well as one *trans* deoxycytidine adduct and a deglycosylated adduct at the N-7 position of guanine.

Using an approach described in a previous report, an adduct corresponding to *trans* opening of the (1*R*,2*S*)-diol (3*S*,4*R*)-epoxide of phenanthrene by the exocyclic amino group of dA was synthesized in activated form and incorporated, by modified solid phase DNA synthesizer methodology, into a nonanucleotide comprising codons 60-62 of the human K-ras b oncogene. In duplexes formed by this adducted oligonucleotide, substitution of a dG for a thymidine residue in the complementary strand opposite the modified dA had almost no effect on the  $T_m$ , suggestive of a possible role for such base-pair "mismatches" in mutations caused by diol epoxide adduct formation.

We previously reported the use of the pS189 shuttle vector containing the *supF* gene to probe the mutagenic specificities of the four configurational isomers of benzo[c]phenanthrene 3,4-diol 1,2-epoxide. We have now compared these mutation hotspots with sites of DNA polymerase arrest when the diol epoxide-modified DNA is used as a template for DNA replication by Sequenase, version 2.0. It was presumed that the presence of a bulky adduct on the template would block

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further extension of the primer chain by the polymerase. Although sites of polymerase arrest tended to be found at 1 or 2 nucleotides 3' to mutation hotspots, arrest bands near hotspots were not particularly prominent in all cases, and there were many sites of polymerase arrest that were not near mutation hotspots. Thus, factors in addition to chemical selectivity for adduct formation appear to contribute to the mutagenic selectivity of these diol epoxides.

## SECTION ON OXIDATION MECHANISMS

### Mechanistic Enzymology of HIV Proteins

Kinetic and Mechanistic Studies of the Autoprocessing of HIV-1 Protease from an Analog of the Gag-Pol Polypeptide Precursor. In a previous report we described a construct containing the HIV-1 protease with flanking Pol region sequences ( $\Delta TF$  and  $\Delta Pol$ ) that was expressed as a fusion protein with the maltose binding protein (MBP) of the *malE* gene of *Escherichia coli*. This protein, MBP- $\Delta TF$ -PR- $\Delta Pol$ , provides a convenient model for the study of autoprocessing of the polypeptide since it contains only two potential protease cleavage sites, one on each side of the sequence (PR) corresponding to the mature protease. Kinetic results previously reported focused on the proteolytic steps leading to mature HIV-1 protease that occur subsequent to renaturation of the denatured full length fusion protein. Of particular interest was the observation that the initial proteolytic cleavage ( $k_{first}$ ), which occurs at the amino terminus of the protease sequence and results in disappearance of the full length fusion protein, appearance of the MBP and development of enzymatic activity comparable to that of the mature protease, is first-order in protein concentration, even though two polypeptide chains are needed to form a catalytically competent active site. Since the protein is initially in denatured form, one possible explanation for the observed first-order kinetics is that a folding process involving the monomeric protein is slower than subsequent autoprocessing steps requiring dimeric protein, and is thus rate limiting. Folding of the full length fusion protein, as followed by fluorescence spectroscopy, involves at least two kinetically distinct steps, the first of which is too fast to measure using conventional mixing techniques. The second step is first-order with respect to protein concentration and has a rate constant ( $k_{fold} = 0.52 \text{ min}^{-1}$ ) that is insensitive to urea concentration. A similar fluorescence change is observed for the folding of the monomeric MBP but not for the dimeric mature HIV-1 protease. We attribute  $k_{fold}$  to cis/trans isomerization of peptide bonds (33 and 22 in MBP- $\Delta TF$ -Protease- $\Delta Pol$  and MBP, respectively) involving the imino nitrogen of proline. Since  $k_{fold}$  is ca. 15 times faster than the initial proteolytic step ( $k_{first} = 0.036 \text{ min}^{-1}$ ; see above), protein folding is not rate-limiting for this process. The likeliest mechanism for the first-order step ( $k_{first}$ ) is thus one in which the fusion protein is predominantly in the dimeric form at the concentrations used, and undergoes intramolecular cleavage.

We have compared the efficiency of intramolecular autoprocessing of the fusion protein MBP- $\Delta TF$ -Protease- $\Delta Pol$  with its intermolecular cleavage catalyzed by the mature HIV-1 protease. As in the intramolecular reaction, the initial intermolecular cleavage occurs at the amino terminus of the protease sequence, as indicated by time-dependent appearance of MBP- $\Delta TF$  (monitored by SDS-PAGE), as well as by accumulation of the PR- $\Delta Pol$  fragment (13.2-kDa protein), which underwent slower conversion to the 11-kDa protease. After a lag period of ca. 3 min that corresponds to the time needed for the folding of the fusion protein, the reaction followed first-order kinetics for at least three half-lives. A plot



of  $k_{obs}$  vs the concentration of mature protease (in 100 mM acetate buffer, pH 5.2, at 25 °C) was linear with a positive intercept ( $[protease] = 0$ ) corresponding to the rate constant for the intramolecular reaction and a slope corresponding to  $k_{cat}/K_m$  for the intramolecular, protease catalyzed reaction. The concentration of mature HIV-1 protease that would produce a reaction rate equal to that of the intramolecular process was calculated to be 1.4  $\mu M$  from the relationship,  $k_{first} = k_{cat}[E_0]/K_m$ . Thus, even though the dimeric full length fusion protein has a much lower intrinsic catalytic activity than the mature enzyme, intramolecular cleavage predominates at low ( $> ca. 1 \mu M$ ) concentrations of mature protease, because of the entropic advantage conferred by attachment of the scissile peptide bond to the catalytic protein. Thus, an intramolecular mechanism must prevail early in the course of autoprocessing.

In collaboration with Dr. John M. Louis [project No. Z01 DK 15503-2 LCDB], the 13.2-kDa protein intermediate, PR- $\Delta$ Pol, observed on the pathway of autoprocessing of MBP- $\Delta$ TF-PR- $\Delta$ Pol to mature protease, has been purified to  $>90\%$  homogeneity. Preliminary results indicate that the dissociation constant of this dimeric protein ( $K_D$ ) is several orders of magnitude larger than those for the mature protease or the full length fusion protein. At a protein concentration above  $K_D$ , the 13.2-kDa protein appears to have catalytic activity comparable to that of the mature (11-kDa) protease.

Kinetics of Nonprocessive Template-Directed Incorporation of 4-Thiothymidine into Oligonucleotides. We are interested in the effect of substitution of sulfur for oxygen in DNA bases on the rates and mechanisms of nucleoside triphosphate incorporation into primers by HIV-1 RT and other DNA polymerases, for two reasons: i) the long wavelength absorption of the sulfur analogs makes them convenient substrates for spectrophotometric measurements of enzymatic reaction rates, and ii) the different steric and hydrogen-bonding characteristics of sulfur vs oxygen suggest that these analogs may provide useful probes for the mechanistic roles of these factors. The 5'-triphosphate of 4-thiothymidine (4-STTP) is a substrate for the Klenow fragment of DNA polymerase I of *Escherichia coli*. Its incorporation into the primer TCGACGCCG that is annealed to the template AAACCTTGGACGGCTGCGA was followed by measuring the decrease in absorption at 335 nm ( $\Delta\epsilon = 6300 M^{-1}cm^{-1}$ ). Comparison of steady-state kinetic parameters for the nonprocessive reaction of 4-STTP with this template/primer (10  $\mu M$  template/primer in phosphate buffer, pH 7.4, 25 °C) with those for reaction of the natural substrate, thymidine triphosphate (TTP) indicates that  $K_m$  for 4-STTP is approximately 3.5 times greater than for TTP, whereas  $k_{cat}$  for 4-STTP is approximately 6 times greater than for TTP. The modest increase in  $K_m$  for 4-STTP may result from the larger size of the sulfur relative to the oxygen atom. The larger increase in  $k_{cat}$  must reflect an increase in the rate of dissociation of the product from the enzyme, since this dissociation step is known to be rate limiting for nonprocessive incorporation of a single nucleotide residue into template/primer.

## SECTION ON PHARMACODYNAMICS

### Adenosine Receptor Agonists and Antagonists

Adenoregulin: Effects on Binding and Function of Receptors. Adenoregulin, an amphiphilic frog peptide markedly enhances binding of agonists to  $A_1$ ,  $A_2$  and 5HT $_{1A}$  receptors in brain membranes. At higher concentrations adenoregulin

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enhances binding of an agonist to  $A_{2a}$  striatal receptors and now begins to inhibit binding to the  $G_i$ -coupled receptors. In the presence of adenosine, the guanyl nucleotide-insensitive binding of agonists now becomes sensitive to GTP or GTPS; i.e., the receptor is converted to a low affinity state by guanyl nucleotides. Thus, the stimulatory effects of adenosine on agonist binding may reflect an enhanced coupling of with receptor  $G_i$ -protein, leading to an increase receptors in the high affinity state. Mastoparan, another amphiphilic peptide, is known for effects on release of transmitters and for stimulation of GTPase activity of the  $G_i/G_o$  class of guanyl nucleotide binding proteins. Mastoparan has similar effects to adenosine on binding of agonists to  $A_1$ ,  $A_2$  and  $5HT_{1A}$  receptors. Conversely, adenosine, like mastoparan, causes increases in calcium influx, transmitter release and phosphoinositide breakdown. Such effects occur at higher concentrations and perhaps correlate with the inhibitory effects of these peptides on receptor binding. No clear correlates of the enhancement of agonist binding with effects on cyclic AMP formation or phosphoinositide breakdown have been obtained. In membranes and permeabilized cells both peptides are inhibitory to phosphoinositide breakdown. Effects on binding of GTPS to isolated brain  $G_i/G_o$  proteins are under study.

Structure Activity Relationships of Agonists and Antagonists at Adenosine Receptors: Various paraxanthine analogs (1,7-disubstituted) and 1,8-disubstituted xanthines have been prepared by new synthetic procedures. Certain of these have high affinity and selectivity for  $A_1$  receptors. Remarkably, the agonist activity for 2-(ar)alkoxyadenosines at  $A_{2a}$  receptors coupled to adenylate cyclase in human platelet membranes does not correlate with the anti-aggregatory activity. There is a strong correlation for a group of 12 other well-characterized adenosine analogs, suggesting that the 2(ar)alkoxyadenosines act differently in platelets from other classes of adenosine analogs. The low affinity  $A_{2b}$  receptor-coupled to adenylate cyclase was characterized in fibroblast membranes and intact cells. Antagonists are more potent in intact cells than in membranes. Only alloxazine appears selective for  $A_{2b}$  receptors, while certain xanthines, in particular a 8-styrylcaffeine, are selective for the high affinity  $A_{2a}$  receptor.

Caffeine Analog and Calcium Release: 1-Propyl-3,7-dimethylxanthine and 1-propargyl-3,7-dimethylxanthine are 4 to 5-fold more potent than caffeine in causing elevation of intracellular calcium, as monitored by fura-2 in pheochromocytoma cells. 1,3,7-Tripropargylxanthine also is more potent, while a wide range of other xanthines are either similar in activity to caffeine or were less potent.

Biochemical and Behavioral Alterations After Chronic Caffeine: Chronic ingestion of caffeine (100 mg/kg/day) by NIH-strain mice results in a significant reduction after about 48 hours in locomotor activity and to alterations in behavioral responses to caffeine, other xanthines and adenosine analogs, the last both alone and in combination with xanthines. Alterations in behavioral responses to cholinergic agents also occur, while effects of chronic caffeine on behavioral responses to dopaminergic agents are minimal. These alterations in behavioral responses are paralleled by changes in receptor densities. Thus, chronic caffeine ingestion increases density of cortical and striatal  $A_1$ -adenosine receptors. Striatal  $A_{2a}$  receptors are unaltered, as are densities of striatal  $D_1$  and  $D_2$ -dopaminergic receptors. The densities of  $5HT_1$  and  $5HT_2$ -serotonergic receptors, nicotinic and muscarinic receptors and benzodiazepine-binding sites on  $GABA_A$  receptors are increased. The densities of MK-801 binding



sites associated with NMDA-glutaminergic receptors appear unaltered. The densities of cortical  $\beta_1$  and cerebellar  $\beta_2$ -adrenergic receptors are reduced, while  $\alpha_1$ - and  $\alpha_2$ -adrenergic receptors are unaltered. Densities of L-type calcium channels are increased, which is also the case after chronic ethanol ingestion. Such biochemical changes may underly the tolerance and withdrawal syndromes caused by chronic caffeine. High doses of caffeine cause choreiform (dance-like) movements in mice and may provide an acute model for certain human choreas. The choreiform movements elicited by caffeine appear related to blockade of  $A_{2a}$  and not  $A_1$  adenosine receptors. Surprisingly haloperidol-sensitive dopamine systems do not appear involved in the caffeine-elicited choreiform movements. The choreas elicited by caffeine are markedly reduced after chronic caffeine ingestion.





ANNUAL REPORT OF THE LABORATORY OF BIOORGANIC CHEMISTRY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE  
AND KIDNEY DISEASES

SECTION ON BIOCHEMICAL MECHANISMS

Analogues of Thyrotropin-Releasing Hormone

Analogues of the tripeptide hormone TRH, in which H-2 or H-4 of the imidazole ring has been replaced by a trifluoromethyl group, show pituitary and cardiovascular activities equal to, or better than, those of the natural peptide in the conscious rat. Thus, the loss of imidazole basicity and the introduction of a bulky substituent at either position seems to be unimportant in ligand recognition. Yet, the imidazole ring cannot be irrelevant to recognition since the 4-nitro analogue shows no pituitary activity but retains full cardiovascular activity. We have demonstrated the duality (or multiplicity) of TRH receptors in brain and pituitary. The further demonstration of separation of activities by our novel analogues offers an opportunity to attempt clinical removal of the set of activities governed by one receptor or another. (Trifluoromethyl)imidazoles show the very unique property of losing HF at mildly alkaline pH to form extremely electrophilic difluorodiazafulvenes. The generation of the latter species within a receptor's ligand-binding site may result in irreversible bond formation with a protein nucleophile in the binding site. At 37°C and pH 8.5, the half-time for fulvene generation from 2-CF<sub>3</sub>-Im-TRH is ca. 30 hr. It is unreasonable, therefore, to search for irreversible binding to pituitary or brain tissue during the period of a normal incubation. Analysis of pK and reactivity data for large number of additionally substituted (trifluoromethyl)imidazoles provided the basis for predicting appropriate extra substituents to give TRH analogues with half-lives ranging from 4 sec to 1.5 yr. We have chosen acetamido as the ideal substituent for our purposes and are now engaged in development of synthetic routes to 4-acetamido-2-trifluoromethyl-Im-TRH. Should this compound show the expected reactivity in binding irreversible to specific TRH receptors in tissue homogenates, it will then be administered to rats and followed by a dose of normal TRH. Strong diminution of either pituitary or CVS activity is expected.

Similar approaches are being used to create selective irreversible inhibitors of histamine H<sub>1</sub> and H<sub>2</sub> receptors.

Antimalarial Agents Based on Bioheterocycles

We have found 2-iodo-L-histidine to be a potent antimalarial agent against drug-resistant *Plasmodium falciparum*. However, the compound is effective in monkeys for only 24-48 hrs; we have shown that inactivation may be the result of nonenzymatic deiodination by any of the sulfhydryl compounds present in tissue or serum. The facts that 2-iodohistidine does not block protein synthesis in the parasite, and that the corresponding 2-chloro and 2-bromo compounds are inactive, led us to speculate that the 2-iodo compound operates by "plugging" one or more holes in the erythrocyte membrane and, thus, depriving the parasite of nonamino acid nutrients obtained by diffusion through such holes. Indeed, the diameter of the holes has been estimated at 0.7 nm, almost exactly the width of 2-iodohistidine. We have, therefore, explored the antimalarial activities of more metabolically stable derivatives of histidine which have ring substituents offering similar steric sizes. New general synthetic methods were devised to provide these series of compounds: 1-R-histidines, 2-R'-histidines, 1-R-2-R'-histidines (and the corresponding histamines), in which R need not equal R' and in which R and R' may be any

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saturated, unsaturated, cyclic, bicyclic, arylalkyl or heteroarylalkyl group, or any of the functionalized derivatives. Regiospecific alkylation of N-1 involves the use of cycloureidohistidine; despite the reduced nucleophilicity at N-1, alkylation occurs readily even with such hindered groups as cyclohexyl and t-butyl. Alkylation at C-2 involves the rarely used Minisci reaction, in which R'COOH is decarboxylated to R' radical by a catalytic amount of argentic ion (regenerated by a peroxy salt). In acidic media, the reaction is remarkably specific for alkylation at C-2 in good yield, providing the most efficient and least expensive method yet developed for the production of 2-alkylimidazoles in general, and 2-alkylhistidines or histamines in particular. In vitro screening of a random sampling of these compounds reveals modest inhibitory activity, but sufficient to support the concept of chemotherapy by membrane plugging, or "permeatherapy." Screening of a large number of these histidine or histamine analogs is in process.

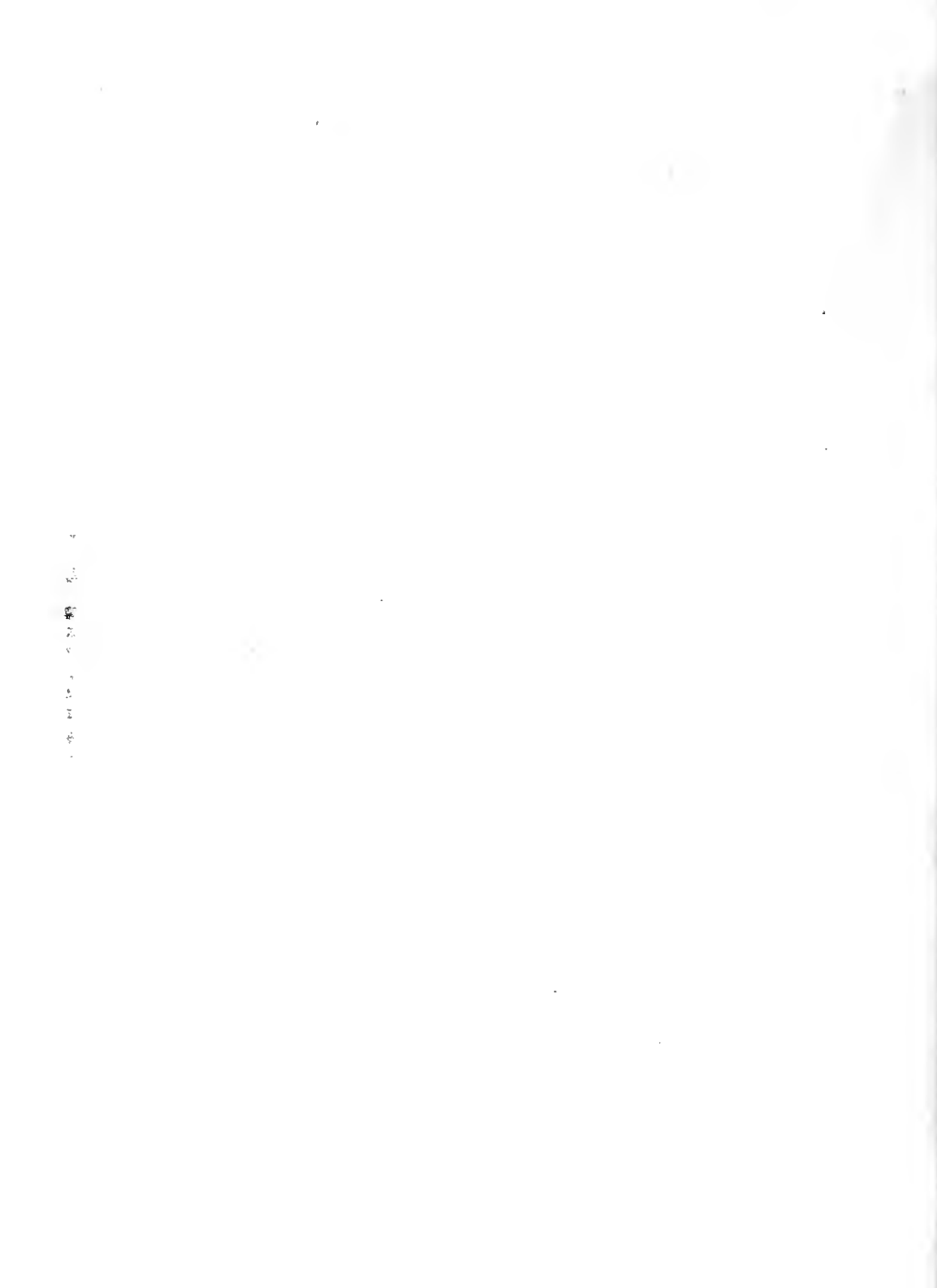
### Stereopopulation Control in Drug Delivery and Enzyme Simulation

We have synthesized a large variety of test-tube models which simulate the enzyme-substrate complex by having the substrate frozen into a single, very favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (stereopopulation control). These compounds undergo intramolecular reactions at rates approaching those catalyzed by enzymes (but independently of any functional assistance). As part of our studies of practical applications of stereopopulation control, we have been exploring the use of various SPC-derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate transport from the gut to the circulatory system to the desired site of action by temporary masking of charge within the molecule, by improvement in lipophilicity and by regeneration based simply on local pH variation in ligand sites or on local concentrations of potent reducing agents. Recent studies have concentrated on o-nitrophenylpropionic acids as carriers. To date, these carriers have been coupled to GABA, to protected DOPA derivatives and to indoleamines. The nitro group is reduced enzymatically, and the resulting amine attacks an amide bond intramolecularly to release the drug. Rapid attack by the amine is ensured by placing a gem-dimethyl group on the adjacent carbon. Kinetic studies have shown that chemical reduction of the nitro group occurs at the same rate with or without the gem-dimethyl group, but that drug release is greatly enhanced by the action of the gem-dimethyl. Surprisingly, xanthine oxidase reduced the hindered nitro compound more readily than the unhindered. This result reveals that the enzyme must be able to reach a face of the nitro group for reduction, and not just an edge. Response to other reducing agents is now being explored with a view to using such prodrugs to deliver mustards and other anticancer agents to hypoxic cells.

Similar model systems have been designed with conformationally frozen indole and phenolic rings, in order to simulate and study the tight charge transfer complexes achieved by tryptophan in proteins or by bioindoles in receptor binding.

### Bioindoles and Oxindoles As Medicinal and Diagnostic Agents

Interest in the physiological activities and potential clinical uses of the pineal hormone melatonin (N-acetyl-5-methoxytryptamine) has grown enormously in recent years. The hormone has been implicated in the regulation of phenomena as diverse as the sleep-wake cycle, depression, sexual urge, neuroimmunomodulation, mammary cancer, etc. We have mounted the first systematic program to develop agonists and antagonists for the hormone, and affinity labels for its multiple receptors. 2-Iodo- and 2-bromomelatonin can now be made by one-step syntheses and bind to the known receptor much more effectively than melatonin itself. Our new and greatly improved syntheses for these compounds will also facilitate preparation of labelled compounds for radioimmune assay. The superior binding implies that the receptor contains a large lipophilic hole, which may



bind other 2-substituted melatonin analogues even more effectively. Synthetic methods are now being developed to obtain such analogues by radical alkylation procedures, which proved so effective in producing 2-alkylhistidines and histamines.

Studies have continued on the use of oxindoles as inhibitors of aldose reductase as a means to control diabetic retinopathy. We have now achieved inhibitory potencies (in vitro) equal to, or greater than, those of the best inhibitors generated by pharmaceutical industry, but have totally eliminated incorporation into the structures of strong allergy-inducing hydantoin. New work involves the development of prodrugs suitable for oral administration, based on the slow release of malonic acid structures from their esters by nonenzymatic hydrolysis.

Several years ago, we made a concerted effort to obtain 2-fluoroindoles by halogen exchange with 2-bromoindoles, but were unsuccessful. Direct fluorination by use of new fluorinating agents has now been found surprisingly successful and such compounds (2-fluoroserotonin, 2-fluoromelatonin) are anticipated to find application as affinity labels, analogues of peptide hormones and PET scanning reagents (since the introduction of radiolabel requires only one step).

## SECTION ON DRUG RECEPTOR INTERACTIONS

### Development of Multifunctional Chemotherapeutic Agents

Amantadine is a well-known antiviral agent which has been commercially available for years under the trade name symmetrel. Its primary use is for the prevention of influenza A infections. It also has been reported that at higher doses, it is active against other viral strains. Its mode of action is thought to be at an early stage in replication, probably at the stage of uncoating. In a rational approach to increasing the effectiveness of amantadine, as well as of other antiviral agents, two molecules of amantadine were connected by a linear bridge containing extremely hydrophilic guanidine residues. Interaction of this connecting bridge with viral cell wall glycoproteins was expected to deliver the amantadine residues to the viral surface, thus greatly increasing the effective concentration of the drug. This and related structures were found to be very active against gram positive and gram negative bacteria, fungi, yeast, and enveloped viruses, including herpes simplex and retroviruses. The analogue incorporating the N-chloro functionality also was extremely potent. The exact mechanism of action of these analogues is unknown. The antiviral effects may be due to interaction with or through the viral envelope.

On the basis of test data obtained from these earlier compounds, extensive structural modifications have been carried out in order to produce compounds having maximum therapeutic indices for potential AIDS chemotherapy. In all, 23 new compounds have been prepared and are currently being evaluated against HIV.

### Synthesis and Biochemistry of Ascorbic Acid Analogues

Following literature procedures, 2,3-isopropylidene-L-gluconic acid methyl ester was converted to 6-bromo and 6-chloro-6-deoxy-2,3-isopropylidene-L-gluconic acid methyl ester. Acid hydrolysis accompanied by rearrangement produced 6-bromo- and 6-chloro-6-deoxy-L-ascorbic acid in low yield, as reported. 6-Iodo- and 6-fluoro-6-deoxy-2,3-isopropylidene-L-gluconic acid methyl ester were prepared by nucleophilic displacement of the corresponding 6-tosyl derivative by halide ion. 6-Iodo- and 6-fluoro-L-ascorbic acid was obtained by acid hydrolysis and rearrangement. An alternative synthesis of 6-fluoro-L-ascorbic acid was based on reaction of 2,3-di-O-benzyl-L-ascorbic



acid with diethylamino sulfur trifluoride (DAST), followed by hydrogenolysis. To date, the 6-chloro- and 6-iodo analogues have been studied as substrates and inhibitors for the ascorbic acid transport of ascorbic acid competitively, and were transported into the cells.

Further biochemical characterization of the 6-halo analogues is in progress. However, the apparent insensitivity of the transport system to substitution at carbon 6 of ascorbic acid has directed our attention to further functionalization at this position in work designed to develop additional functionalized analogues. 2,3-Di-O-benzyl-L-ascorbic acid was activated by conversion of the 5,6-epoxy derivative using the Mitsunobu reaction. Attempted nucleophilic displacement by basic nucleophiles led to carbanion formation at carbon-4 and intramolecular epoxide opening to produce 2,3-di-O-benzyl-4,5-dehydroascorbic acid. This provided a fortuitous route to 5-deoxy-D,L-ascorbic acid since catalytic debenzoylation also led to reduction of the 4,5-double bond. Reaction of the epoxide with aromatic amines gives clean addition, presumably to the 2,3-di-O-benzyl-6-arylamino-6-deoxy analogues. Characterization of these products is in progress. Attempted debenzoylation has led to unstable product, possibly as a result of intramolecular reactions between the amine and the electrophilic carbon-3. The epoxide also reaction with higher order organocuprates, albeit in low yield due to intramolecular epoxide opening described above. 4,5-Isopropylidene-L-ascorbic acid also was found to inhibit ascorbate transport, although a relatively higher concentrations. Preparation of functionalized 4,5-benzylidene analogues is in progress.

#### Biological Properties of Fluorinated Neuroactive Amines

Interactions of the benzylic OH group and the fluoro substituent have been considered as contributing factors to explain the effects of fluorine substitution on the adrenergic selectivities of adrenergic agonists. We have previously investigated fluorinated analogues of three types of agonists that have significantly different juxtapositions of a side-chain OH group and the aromatic fluorine, including the natural phenethanolamine agonists (norepinephrine [NE] and epinephrine [EPI]), 3-amino-1-(3,4-dihydroxyphenoxy)-2-propanol, and 3-amino-2-(3,4-dihydroxyphenyl)-1-propanol, wherein the benzyl OH is replaced with the hydroxymethyl group. As in the natural agonists, norepinephrine and epinephrine, substitution of fluorine in the 6-position of the aromatic ring caused a decrease in beta-adrenergic potency in the phenoxypropanolamine and, to a lesser extent, in the hydroxymethyl analogue. Two factors prompted us to extend these studies to 2- and 6-fluoro analogues of 2-(3,4-dihydroxyphenyl)morpholine. Foremost was the realization that, unlike phenoxypropanolamine and hydroxymethyl analogues, with the morpholine analogues we would be able to examine effects of fluorine substitution in a series that possesses both alpha- and beta-adrenergic activity. In addition, while we feel comparable intramolecular interactions of the aromatic fluorine with the benzylic hydroxyl groups of NE, and the side-chain hydroxyl groups of 3-amino-1-(3,4-dihydroxyphenoxy)-2-propanol and 3-amino-2-(3,4-dihydroxyphenyl)-1-propanol, we have no direct evidence for this. In fact, one of the explanations for the potent beta-adrenergic activity of involves a folded conformation that places the propanolamine hydroxyl group in the same position as the ethanolamine hydroxyl group of NE. Unlike our previous examples, 2-(3,4-dihydroxyphenyl)morpholine has no aliphatic side chain hydroxyl group. We note that fluorine substitution had little to no effect on the relatively weak but significant adrenergic activities of dopamine, a fact that led us initially to propose that intramolecular interactions involving the benzylic hydroxyl group of the ethanolamine agonists could be important.

In contrast to our results obtained from our previous studies, fluorine substitution has little effect on the affinities of these morpholine analogues at either alpha- or beta-adrenergic receptors. Thus, in the two series we have examined wherein no hydroxyl group is present--2-(3,4-dihydroxyphenyl)morpholine and dopamine--fluorine has little or





no effect on adrenergic activity. Considering the lack of adrenergic selectivities of fluorodopamines, we previously suggested that electronic effects of specific binding interactions of the aromatic ring might not be significant if the additional securing of the agonist to the binding site by the benzylic hydroxyl group were absent. We presently are developing strategies to try to answer new questions that have been raised by these results.

### Effect of pH on Adrenergic Activity of Fluorinated Norepinephrine

Our initial interest in the preparation of ring-fluorinated analogues of norepinephrine, and of other biogenic amines, was prompted in part by the expectation that fluorine-induced lowering of phenolic pKa's would alter biological properties of these analogues. However, observed adrenergic selectivities seen fluorinated adrenergic agonists appear not to be related directly to altered phenolic pKa's. The minimal changes in adrenergic activity seen with 5-fluoronorepinephrine (5-FNE), and the minimally altered phenol acidity of 6-FNE illustrate this point. Nonetheless, the relationship between ionization and activity of sympathomimetic amines has been the subject of many investigations over several decades. In a recent study, IJzerman et al. measured beta-adrenergic affinities as a function of pH and concluded that the cationic form of catecholamines (amine protonated, phenol unionized) interact with the beta-adrenergic receptor. To examine more closely the effects of phenol ionization on adrenergic activity, we have determined binding affinities of a series of fluorinated analogues of NE at alpha-1, alpha-2, beta-1, and beta-2-adrenergic receptors at three pH values (pH 6.0, acidic; pH 7.4, physiological; pH 8.4, basic). An additional goal of this study was to determine if fluorine-induced adrenergic selectivities would be influenced by changes in pH of the media (spectrophotometric estimates of phenol acidities of FNE's and NE--2-F: 7.8; 5-F: 7.9; 6-F: 8.5, 2,5-DiF: 7.5, NE, 8.9).

All compounds tested showed a significant decrease in affinity at pH 8.4 for alpha-2, beta-1, and beta-2 adrenergic receptors, consistent with the proposal of IJzerman et al. that the cationic form of phenolic amines is required for beta-adrenergic receptor binding. A dramatic drop in binding of 5-FNE and 2,5-DiFNE at pH 8.4 gives especially compelling confirmation that ionization of the phenol is detrimental to binding. In contrast, affinities of the NE and the fluorinated ligands for the alpha-1 adrenergic receptor showed an increase in binding affinity in going from pH 7.4 to pH 8.4. Although we have no explanation for this unexpected result, we are analyzing amino acid sequences of the four receptor sub-types with the hope of finding a charged residue, conserved in alpha-1 adrenergic receptors but absent in alpha-2, beta-1, and beta-2 adrenergic receptors, that could participate in binding at high pH values.

A final point pertains to fluorine-induced selectivities. There appears to be no dramatic pH-related changes in selectivities of 2-FNE for beta-adrenergic receptors, and 6-FNE for alpha-adrenergic receptors. An apparent increase in beta-selectivity of 2-FNE relative to alpha-1 receptors seen at pH 8.4 reflects the more pronounced decrease in binding of the fluorinated analogue at alpha-1 adrenergic receptors relative to NE. For example, at pH 8.4, NE is 79 fold more potent than 2-FNE at the alpha-2 receptor, compared to 55-fold and 7-fold greater potencies at pH 6.0 and 7.4 respectively. In contrast, at the alpha-1 adrenergic receptor, 2-FNE has significant binding at pH 8.4, in contrast to its behavior at lower pH.

### Fluorinated Analogues as [<sup>18</sup>F]-PET Scanning Probes

[<sup>18</sup>F]-6-FluoroDOPA, functioning as a precursor for [<sup>18</sup>F]-6-fluorodopamine, is used as a PET scanning agent for central dopaminergic function. Likewise, [<sup>18</sup>F]-6-fluorodopamine, functioning in the periphery as a precursor for [<sup>18</sup>F]-6-fluoro-



norepinephrine and incapable of crossing the blood-brain barrier, is used as a PET scanning agent for peripheral noradrenergic function. To date, there is available no comparable PET-scanning agent for central noradrenergic function. We are exploring the synthesis of 6-fluoro-3-threo-dihydroxyphenyl serine (6-F-3-threo-DOPS) as a precursor for 6-fluoronorepinephrine that can cross the blood brain barrier. Since biosynthesis of 6-fluorodopamine in the CNS would be circumvented, [ $^{19}\text{F}$ ]-6-FNE resulting from decarboxylation of [ $^{18}\text{F}$ ]-6-F-3-threo-DOPS could be taken up and stored in central adrenergic neurons for PET visualization. In initial studies, we have used an enantioselective synthesis to prepare 6-F-3-threo DOPS from 6-fluoroveratraldehyde.

### Three-dimensional Structure of Muscarinic Receptors

Current models of the three-dimensional structure of muscarinic and other G protein-coupled receptors are primarily based on high-resolution structural data obtained for bacteriorhodopsin, whose molecular structure is characterized by the presence of seven alpha-helical transmembrane domains (TM I-VII). To provide more direct insight into the molecular structure of muscarinic receptors, we have analyzed the pharmacological properties of a series of hybrid m2/m5 muscarinic receptors. Initially, we identified several chimeric constructs which, upon transient expression in COS-7 cells, were unable to bind significant amounts of the radioligand [ $^3\text{H}$ ]N-methylscopolamine. A common structural feature of these constructs was the presence of m2 receptor sequence in TM VII and of m5 receptor sequence in TM I. The ligand binding activity of these "pharmacologically inactive" hybrid receptors could be restored by replacing TM I (containing m5 receptor sequence) with the corresponding m2 receptor domain. These data provide the first direct experimental evidence that the molecular architecture of muscarinic receptors (and, most likely, that of other G protein-coupled receptors) resembles that of bacteriorhodopsin in that the seven TM helices are arranged in a ring-like fashion such that TM I lies directly adjacent to TM VII.

### Acetylcholine Binding Domain

Pharmacological analysis of a series of m3 receptor point mutants has identified several Thr and Tyr residues which appear to be critically involved in acetylcholine (ACh) binding. These residues (which are conserved among all muscarinic receptors, but, with very few exceptions, are not present in other G protein-coupled receptors) are positioned at a similar level (one to two helical turns away from the membrane surface) within different TM helices (TM III, V, VI, and VII) and are predicted to define the ACh binding domain. Binding studies with a series of ACh derivatives suggest that most of the conserved Thr and Tyr residues interact with the ACh ester group by means of hydrogen bonding.

### Residues Involved in Receptor Activation

Functional studies with Thr→Ala and Tyr→Phe mutant m3 receptors showed that Thr234 and Tyr506 (rat m3 receptor sequence) are required for efficient agonist-induced receptor activation. These two residues are located at a similar level within TM V and VI, respectively. These two TM helices are connected by the third intracellular loop (i3), a region known to play a pivotal role in G protein activation. One may speculate that Thr234 and Tyr506 (which are conserved among all muscarinic receptors) are involved in the agonist-induced conformational changes that trigger the activation of the i3 domain and the interaction with specific G proteins.

### Residues Involved in G Protein Coupling



We have previously shown that the N-terminal portion of the i3 loop is of pivotal importance for G protein recognition and activation. The functional significance of individual amino acids contained in this sequence element was studied by systematic mutational modification of the rat m3 receptor. Functional studies identified a single amino acid within the N-terminus of the i3 domain (Tyr254; rat m3 receptor sequence) which is essential for efficient receptor-mediated activation of the PI pathway. This Tyr residue is conserved among most biogenic amine and glycoprotein hormone receptors, suggesting that it may play an important functional role also in other G protein-coupled receptors.

### Role of Amino Acids Conserved Among Most G Protein-Coupled Receptors

Virtually all G protein-coupled receptors contain within their seven TM domains several highly conserved Pro and Trp residues. To study their potential functional roles, a series of mutant m3 receptors in which the conserved Pro and Trp residues were individually replaced with Ala and Phe, respectively, were created and pharmacologically characterized in COS-7 cells. Whereas less pronounced changes in receptor function resulted from the Trp->Phe substitutions, dramatic changes in ligand binding affinities, receptor densities, and receptor function were observed with the four Pro->Ala mutant receptors studied. Our data indicate that the conserved Pro residues play key roles in the maintenance of a stable protein fold and the process of ligand-induced receptor activation.

### Subunit Character of Muscarinic Receptors and Possible Functional Implications

Truncated forms of the m2 and m3 muscarinic receptors (containing TM I-V and the N-terminal portion of i3) were found to be functionally inactive and did not bind muscarinic ligands. However, when the truncated receptors were coexpressed in COS-7 cells with the corresponding C-terminal receptor domains (containing TM VI and VII and adjacent intra- and extracellular sequences), functional muscarinic receptors were obtained. This finding suggests that muscarinic receptors (and possibly other G protein-coupled receptors) behave in a fashion analogous to two-subunit receptors.

To test the hypothesis that the association between the two structural muscarinic receptor subunits may occur not only intra- but also intermolecularly (thus providing a molecular basis for receptor dimerization), coexpression experiments were carried out with different mutant m3 and chimeric alpha2-adrenergic/m3 muscarinic receptors. None of these mutant receptors was able to efficiently couple to G proteins mediating stimulation of PI hydrolysis when expressed alone. In contrast, considerable functional activity could be observed in the coexpression experiments. These results suggest that muscarinic receptors (and possibly other G protein-coupled receptors) are able to interact functionally with each other at a molecular level. The possible physiological importance of this phenomenon clearly deserves further investigations.

### Functionalized Congeners of Bioactive Compounds

By the functionalized congener approach to drug design, new analogs are synthesized with the regiospecific inclusion of a functionalized chain at a point which can accommodate molecular modification and a certain degree of steric bulk. The resulting functionalized drug congener may then be attached through an amine or other reactive group on the chain to various organic moieties, such as amines and peptides. The receptor-binding affinity of these analogs is often greater than that of the parent drug and does not necessarily diminish as the molecular weight is systematically increased. APEC (2-(aminoethylaminocarbonyl)ethylphenylethylamino)-5'-N-ethyl-carboxamidoadenosine), a high affinity A2a-adenosine agonist, was designed by this approach.



Applications of the functionalized congener approach include the synthesis of probes for receptor studies (for example: radioligands, fluorescent probes, or chemically reactive affinity labels) and the design of drug delivery systems (including targeting and altering the characteristics to allow for passage through membranes). Drug conjugates may be designed in a stepwise approach to optimize certain pharmacological properties, such as potency, specificity, and duration of action.

Functionalized congeners are also being explored for therapeutic goals. Most clinically available cholinergic drugs (e.g. the agonist pilocarpine and the antagonist atropine) are non-selective in their interaction with muscarinic receptor subtypes. The recent cloning, sequencing, and expression of five separate genes for muscarinic receptors has raised the possibility of developing novel organic compounds that act as agonists or antagonists at one of these subtypes. Selective compounds could be therapeutically useful in treating a variety of diseases, including Alzheimer's disease, cardiac disease, neurogenic bladder, and certain sleep disorders. Furthermore, such specific compounds, by virtue of their subtype selectivity, should be devoid of many of the side effects of currently used compounds.

In view of the above, a goal is to develop novel and selective muscarinic antagonists. We have used a functionalized congener approach in the design of derivatives of telenezepine. These derivatives contain prosthetic groups for radioiodination, protein cross-linking, photoaffinity labeling, fluorescent labeling, and biotin for avidin complexation. We explored the effect of chain length on aryl isothiocyanate derivatives that were found to be receptor affinity labels. The affinity for muscarinic receptors in rat forebrain (mainly m1 subtype) was determined in competitive binding assays vs. [3H]N-methylscopolamine. An isothiocyanate derivative for affinity labeling was found to react irreversibly with forebrain muscarinic receptors. The high affinity of these derivatives make them suitable for the characterization of muscarinic receptors in pharmacological and spectroscopic studies, for peptide mapping, and for histochemical studies.

The ligand recognition site of A2a-adenosine receptors in rabbit striatal membranes was probed using non site-directed labeling reagents and specific affinity labels. Exposure of membranes to diethylpyrocarbonate (DEP) at a concentration of 2.5 mM followed by washing was found to inhibit the binding of [3H]CGS 21680 and [3H]XAC to A2a receptors, by 86 and 30%, respectively. Protection from DEP inactivation by an adenosine receptor agonist, 5'-N-ethylcarboxamidoadenosine, and an antagonist, theophylline, suggested the presence of two histidyl residues on the receptor, one associated with agonist binding and the other with antagonist binding. Binding of [3H]CGS 21680 or [3H]XAC was partially restored following incubation with 250 mM hydroxylamine, further supporting histidine as the modification site. Preincubation with disulfide reactive reagents, DTT or sodium dithionite, at > 5 mM inhibited radioligand binding, indicating the presence of essential disulfide bridges in A2a-receptors, while the concentration of mercaptoethanol required to inhibit binding was > 50 mM.

A number of isothiocyanate-bearing affinity labels derived from the A2a-selective agonist APEC were synthesized and found to inhibit A2a-receptor binding in rabbit and bovine striatal membranes. Binding to rabbit A1-receptors was not inhibited. Preincubation with the affinity label p-DITC-APEC (100 nM) diminished the Bmax for [3H]CGS 21680 binding by 71%, and the Kd was unaffected, suggesting a direct modification of the ligand binding site. Reversal of p-DITC-APEC inhibition of [3H]CGS 21680 binding with hydroxylamine suggested that the site of modification by the isothiocyanate is a cysteine residue. A bromoacetyl derivative of APEC was ineffective as an affinity label at submicromolar concentrations.





Extracellular ATP has a role as a fast cotransmitter that is released in conjunction with norepinephrine and other transmitters at the neuroeffector junctions of many vascular and visceral smooth muscles. Recently, it was reported that ATP can act as a fast transmitter at synapses between neurons in the coeliac ganglion and in the central nervous system via opening of ligand-gated ion channels. Study of P2-purinoceptor subtypes has been difficult due to the lack of potent and selective ligands.

With the goal of developing high affinity P2-purinoceptor-selective agonists, we have synthesized a series of analogues of adenine nucleotides modified on the purine ring as chain-extended 2-thioethers or as N6-methyl substituted compounds. Chemical functionality incorporated in the thioether moiety included cyanoalkyl, nitroaromatic, amino, thiol, cycloalkyl, n-alkyl, and olefinic groups. Apparent affinity of the compounds for P2Y-purinoceptors was established by measurement of P2Y-purinoceptor-promoted phospholipase C activity in turkey erythrocyte membranes and relaxation of smooth muscle in three different preparations (guinea pig taenia coli, rabbit aorta, and rabbit mesenteric artery). Increases in intracellular calcium in response to P2Y-agonists were measured in rabbit myotubes. Activity at P2X-purinoceptors was established by measurement of contraction of rabbit saphenous artery and of the guinea pig vas deferens and urinary bladder. The P2Y-potencies of certain long chain 2-thioethers were enhanced over 2-methylthioATP.

In an attempt to develop new methods for screening drugs at P2y purinergic receptors, we attempted to use [35S]ADP-beta-S to label purinergic receptors in bovine brain. Fresh bovine brain membrane preparations were shown to degrade ATP rapidly, but this nucleotidase activity was found to decline upon prolonged storage of the membranes at -80 °C and was abolished after a few weeks. [35S]ADP-beta-S binding to bovine brain membranes that had been stored for a minimum of 6 weeks was saturable ( $K_d$  14 nM,  $B_{max}$  6.8 pmol/mg protein) and was best described by a one-site model. [35S]ADP-beta-S binding was displaceable by a large number of compounds known to interact with P2 receptors, as well as by various related nucleotides. However, the profile was not consistent with the SAR of P2y receptors, or any other well-defined subtype of P2 receptors.

#### Prosthetic Groups for Labeling of Functionalized Drugs and Peptides

Binding sites for certain drugs in an animal or organ may be localized as a result of the synthesis of high specific activity radiolabelled analogs which have high affinity for that binding site. Prosthetic groups may be attached to a drug or other receptor ligand for the purpose of efficient and selective chemical capture of a particular radioisotope. Having developed functionalized congeners of theophylline and other drugs acting at adenosine receptors, we are now developing prosthetic groups for radioisotopes such as  $^{18}F$ ,  $^{123}I$ , and  $^{125}I$ , to be coupled to these functionalized drug molecules. The prosthetic groups contain amino or carboxylic groups which are to be condensed covalently to functionalized drugs to give conjugates of high affinity at a particular receptor. These prosthetic groups contain amino or carboxylic groups which are to be coupled covalently to functionalized drugs to give conjugates of high affinity at a particular receptor.

We have synthesized new adenosine receptor ligands using a "functionalized congener approach", by which potential positions for attachment of chains on a pharmacophore are empirically probed. The site of attachment must correspond to a region of relaxed steric requirements at or near the receptor binding site. This strategy has allowed us to target accessory sites of favorable interaction on the receptor, and actually enhance the affinity of the ligands. We have used such congeners to make fluorescent probes, super-potent lipid conjugates, affinity labels, etc.



A prosthetic group methodology was utilized to achieve both radioiodination and receptor affinity labeling. In collaboration with Dr. Gary Stiles of Duke University Medical Center, A2a receptors have been labeled using the iodinated agonist PAPA-APEC (Figure 1), suggesting a molecular weight of 45,000 Daltons (bovine striatum). The prosthetic group for radioiodination, PAPA (p-aminophenylacetic acid), was coupled to the functionalized congener APEC. Photoaffinity labeling of canine striatal A2a receptors revealed a single band at 34 kDa. Also, antibodies were raised to the second extracellular loop of A2a receptors and to the long C-terminal tail found in the A2a but not in the A1 receptor. After deglycosylation the receptor still bound ligands and antibodies appropriately. The A2a receptor of the canine liver was immunologically distinct from the brain receptor, thus they may represent distinct subtypes.

The fluorescein conjugate, FITC-APEC (2-[4-[2-[2-[1,3-dihydro-1,1-bis(4-hydroxyphenyl)-3-oxo-5-isobenzofuranthioureidyl]ethylamino-carbonyl]ethyl]phenyl]-ethylamino]-5'-N-ethylcarboxamidoaden-osine), is a novel ligand derived from a series of functionalized congeners that act as selective A2-adenosine agonists. The binding of FITC-APEC to bovine striatal A2-adenosine receptors measured by fluorescence techniques was saturable and of high affinity with a Bmax of 2.3 pmol/mg protein and KD of 57 nM. The KD value estimated by fluorescence was consistent with the Ki of 11 nM obtained in competition studies versus [3H]CGS 21680. FITC-APEC exhibited rapid and reversible binding to bovine striatum. The potencies of chemically diverse A2-adenosine receptor ligands estimated by inhibition of FITC-APEC binding were in good agreement with their potencies determined using radioligand binding techniques. FITC-APEC binding was not modulated by purine derivatives that do not recognize A2-adenosine receptors. These findings validate the use of the novel fluorescent ligand, FITC-APEC, to quantitatively characterize binding to A2-adenosine receptors.

#### Development of Drugs Acting at Adenosine Receptors

Adenosine acts as a neuromodulator in the circulatory, endocrine, immune and central nervous system. The biological activity is associated with two receptor subtypes: the A1-adenosine receptor mediates cardiac and central depressant and antipolytic activities and is coupled to adenylate cyclase in an inhibitory manner; the A2-receptor is involved in vasodilation and antithrombotic functions, possibly through stimulation of adenylate cyclase. The major class of antagonists, the alkylxanthines, generally acts at both receptor subtypes; there is currently a search for analogs which have high potency, high receptor subtype selectivity, and increased water solubility.

Adenosine acts as a neuromodulator through at least two receptor subtypes, A1 and A2. A2 receptors have been further divided into A2a (high agonist affinity, in striatum) and A2b (low agonist affinity, in fibroblasts) receptors. A number of effector mechanisms (cAMP, PI, ion channels) are activated by adenosine, the best known being adenylate cyclase (inhibited by A1 and activated by A2). Several developments within the past few years have enabled a rigorous examination of the molecular structure and regulation of adenosine receptors: 1) the synthesis of adenosine derivatives, as agonists, and xanthine and non-xanthine antagonists with receptor subtype selectivity; and 2) the cloning of both A1 and A2a adenosine receptors. Our goal is to define the parameters of the binding and receptor activation by purines using a variety of means, including synthetic, genetic, spectroscopic, and computational methodology.

It would be highly desirable to have a general, high affinity A2-antagonist probe for receptor characterization. At this time, several xanthines, such as XAC (a xanthine amine congener) may serve as radioligands at striatal A2-receptors, but this high affinity binding



is limited to certain species in which XAC is not A1-selective. We have explored the structure activity relationships of 8-styrylxanthines at adenosine receptors and found highly A2-selective antagonists. 1,3,7-Trimethyl-8-(3-chlorostyryl)xanthine (CSC) was a moderately potent ( $K_i$  vs. [3H]CGS 21680 was 54 nM) and highly A2-selective (520-fold) adenosine antagonist. 1,3,7-Trimethyl-8-(3-succinoylaminoethylstyryl)xanthine was highly A2-selective (250-fold) and of enhanced water solubility (max. 19 mM). 1,3-Dipropyl-7-methyl-8-(3,5-dimethoxystyryl)-xanthine was a potent ( $K_i$  24 nM) and very A2-selective (110-fold) adenosine antagonist.

In reversing agonist effects on adenylate cyclase, CSC was 22-fold selective for A2a receptors in rat pheochromocytoma cells (KB 60 nM) versus A1 receptors in rat adipocytes. Administered i.p. in NIH mice at a dose of 1 mg/kg, CSC shifted the curve for locomotor depression elicited by the A2a-selective agonist APEC to the right (ED50 value for APEC shifted from 20  $\mu$ g/kg i.p. to 190  $\mu$ g/kg). CSC had no effect on locomotor depression elicited by an ED50 dose of the A1-selective agonist CHA. CSC alone at a dose of 5 mg/kg stimulated locomotor activity by 22% over control values. Coadministration of CSC and the A1-selective antagonist CPX, both at non-stimulatory doses, increased activity by 37% ( $p < 0.001$ ) over CSC alone, suggesting a behavioral synergism of A1- and A2-antagonist effects in the CNS. This offers an hypothesis to explain why caffeine is a psychomotor stimulant, i.e. due to synergism of blocking both receptor subtypes.

Isothiocyanate derivatives of adenosine have been developed as selective affinity labels for A1- and A2-adenosine receptors [Jacobson et al, 1992b]. An amine functionalized congener, APEC (2-[(2-aminoethylamino)carbonyl ethyl phenyl-ethylamino]-5'-N-ethylcarbox-amido adenosine), is a selective agonist at A2a-adenosine receptors. As a means of covalently inhibiting this receptor binding site, APEC was coupled to m- and p-phenylene-diisothiocyanate (DITC). The resulting isothiocyanate derivatives in preincubation with rabbit or bovine striatal membranes irreversibly inhibited radioligand binding at A2a- but not A1-receptors. Inhibition was prevented by theophylline (antagonist) or NECA (agonist). p-DITC-APEC (100 nM) diminished the  $B_{max}$  for [3H]CGS 21680 binding by 71% ( $K_d$  unaffected), suggesting a direct modification of the ligand binding site. Selective inhibitors are potentially of interest in studies of the physiological role of adenosine receptors.

8-(3-Isothiocyanatostyryl)caffeine (ISC) was synthesized and shown to inhibit selectively the binding of [3H]CGS 21680 (an A2a-selective agonist) at adenosine receptors in striatal membranes. The  $K_i$  value at A2a-receptors was found to be 110 nM (rat), with selectivity ratios for A2a versus A1-receptors in rat, guinea pig, bovine and rabbit striatum of >100-fold. Preincubation of membranes with ISC caused a dose-dependent, irreversible antagonism of the binding of [3H]CGS 21680, with an IC50 value of 3  $\mu$ M. The irreversibility is likely due to the presence of the chemically reactive isothiocyanate group, since the binding of the corresponding analogue in which the isothiocyanate was replaced with a chloro group was completely reversible. The potency of ISC to irreversibly inhibit the binding of [3H]CGS 21680 in several species varied in the order rat  $\approx$  guinea pig > bovine  $\approx$  rabbit. In all four species, binding of the A1-selective agonist [3H]R-N6-phenylisopropyladenosine was not diminished by pre-treatment with 2  $\mu$ M ISC. The kinetics of irreversible inhibition of rat A2a-receptors by 2  $\mu$ M ISC gave a  $t_{1/2}$  of approximately 3 min. Following partial inactivation, the remaining rat A2a-binding sites retained the same  $K_d$  value as in control membranes for saturation by [3H]CGS 21680. Thus, ISC appears to be a selective affinity label for A2a- versus A1-receptors in the brain.

Detailed amino acid sequence analyses of A1- and A2a-adenosine receptors were assembled by analogy to other G-protein coupled receptors. The models have been



correlated with pharmacological observations with both native and transiently expressed mutants of bovine A1 receptors in which either His251 or His278 residues have been substituted with Leu. Sites for phosphorylation, palmitoylation, and sodium binding have been proposed. These models conform with the seven trans-membrane domain topology commonly found for receptors linked to G-proteins. Asparagine residues that are potential glycosylation sites are located on E-II in both the A1 and A2 sequences. The A1 and A2 sequences display a number of consensus patterns for phosphorylation by PKA, PKC or by casein kinase II (CK2). The carboxyl terminus of the A2 receptor is rich in serine and threonine, suggestive of phosphorylation by bARK. Many G-protein linked receptors contain a cysteine residue in the C-terminal tail close to H-VII, that is palmitoylated and essential for G-protein. The role of an analogous cysteine residue present in the carboxy tail of the A1 receptor (Cys309), but not the A2 receptor, is yet to be determined. The sequence [SN]-L-A-x-[AT]-D occurs near the cytoplasmic end of H-II in both A1 and A2 receptors and in many other G-protein linked receptors, where it is important in the allosteric modulation of agonist binding by Na<sup>+</sup>. The inhibition of A2-radioligand binding by the histidyl-modifying reagent diethylpyrocarbonate suggested the involvement of His residues in interactions with adenosine agonists and antagonists. We have shown that radioligand binding to A2 receptors is disrupted following treatment with the disulfide reactive reagents mercaptoethanol (>50 mM) or DTT or dithionite (>10 mM). This suggests that disulfide linkages indeed are involved in maintaining the structural integrity of the A2 receptor.

In collaboration with Dr. Ad IJzerman we have recently devised a preliminary molecular model for the binding of adenosine to the A1 receptor, that is consistent with the observed pharmacology, SAR, and calculated ligand conformation. This model may be used to predict sites for interaction between specific amino acid residues of the receptor and its ligands, and may be tested in studies of mutagenesis of the receptor. Eventually the objective will be to synthesize improved ligands, including chemical affinity labels and possibly ligands of high selectivity for other subtypes (e.g. A2b), consistent with the model.

5'-Ester derivatives of the potent adenosine agonists N6-cyclopentyladenosine (CPA) and ADAC were designed as prodrugs for the activation of A1-adenosine receptors *in vivo*. Both alkyl esters or carbonates (designed to enter the brain by virtue of increased lipophilicity) and 1,4-dihydro-1-methyl-3-[(pyridinylcarbonyl)oxy]esters designed to concentrate in the brain by virtue of a redox delivery system were synthesized. In the 5'-blocked form the adenosine agonists displayed highly diminished affinity for rat brain A1-adenosine receptors in binding assays. A dihydropyridine prodrug was active in an assay of locomotor depression in mice, in which adenosine agonists are highly depressant. The behavioral depression was not reversible by peripheral administration of a non-CNS active adenosine antagonist. In an assay of the peripheral action of adenosine, the inhibition of lipolysis in rats, the parent compounds were highly potent, and the dihydropyridine prodrug was much less potent.

Adenosine agonists that do not cross biological membranes, such as the blood brain barrier, were designed for use as *in vivo* probes. A series of N6-p-sulfophenylalkyl and sulfoalkyl derivatives of adenosine was synthesized, revealing that N6-(p-sulfophenyl)adenosine (SPA) is a moderately potent and A1-selective (120-fold) adenosine agonist, of exceptional aqueous solubility of > 1.5 g/ml (3 M). It was very potent in inhibiting synaptic potentials in gerbil hippocampal slices with an IC50 of 63 nM. At a dose of 0.1 mg/kg *i.p.* in rats, SPA inhibited lipolysis (a peripheral A1 effect) by 85% after 1 h. This *in vivo* effect was reversed using a peripherally selective A1-antagonist. The same dose of SPA in NIH Swiss mice (*i.p.*) was nearly inactive in locomotor depression, an effect that has been shown to be centrally mediated when elicited by lower doses of





other potent adenosine agonists, such as N6-cyclohexyladenosine (CHA). HPLC studies of biodistribution of other homologues revealed no detectable drug in the brain (detection limit < 0.1% of plasma level). Although SPA at doses > 0.1 mg/kg in mice depressed locomotor activity, this depression was unlike the effects of CHA and was reversible by peripherally selective antagonists.



PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacologically Active Compounds from Amphibians and Other Natural Sources

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	J.W. Daly	Chief	LBC, NIDDK
Others:	C.R. Creveling	Research Chemist	LBC, NIDDK
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	H.M. Garraffo	Visiting Associate	LBC, NIDDK
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COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

4.4

PROFESSIONAL:

3.9

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Natural products provide a wide range of biologically active agents, many of which have unique profiles of pharmacological activity and therapeutic potential. Over three hundred alkaloids have been identified in extracts from amphibian skins. These included batrachotoxins, which are potent activators of sodium channels, histrionicotoxins, which are noncompetitive blockers of nicotinic receptor channel complexes and of potassium channels, and pumiliotoxins, which have myotonic and cardiotoxic activity due to inhibitory effects on closing of sodium channels. Further alkaloids included 2,5-disubstituted decahydroquinolines, 3,5-disubstituted indolizidines, 5,8-disubstituted indolizidines, 1,4-disubstituted quinolizidines, 3,5-disubstituted pyrrolizidines, pumiliotoxins, homopumiliotoxins and alloppumiliotoxins, and tricyclic alkaloids, including pyrrolizidine oximes, pseudophrynamines, cyclopenta[b]quinolizidines, coccinellines, and the potent analgetic epibatidine. Most of these alkaloids have been detected and characterized from neotropical dendrobatid frogs. Pumiliotoxins, quinolizidines, indolizidines, pyrrolizidines and decahydroquinolines have now been characterized from bufonid toads of the genus *Melanophryniscus* and in mantellid frogs of the genus *Mantella* endemic to Madagascar. The latter contain a new class of alkaloids whose proposed dehydropumiliotoxin structure is being probed by synthesis. Pyrrolizidine oximes and coccinellines are derived in dendrobatid frogs from dietary insects by an extremely efficient alkaloid uptake process. Pyrrolizidine oximes and the potent analgetic epibatidine have been synthesized and are undergoing pharmacological study. Alkaloids, detected for the first time from birds, now include homobatrachotoxin, an anesthetic-like alkaloid, and an alkaloid with behavioral stimulant properties.

THE UNIVERSITY OF CHICAGO

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31101-24LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacology and Metabolism of Biogenic Amines and Related Compounds

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: C. R. Creveling, Research Chemist, LBC, NIDDK

Others:

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Bioorganic Chemistry

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Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The biochemistry, physiology, and pharmacology of biogenic amines, amino acid precursors and metabolic products, and various synthetic derivatives have been investigated. The general areas of study include the effects of fluorine substitution of the properties of biogenic amines, adrenergic antagonists and amino acids and catechol-O-methyltransferase (COMT). Studies on COMT include: 1) immunohistochemical localization and induction of COMT in the luminal epithelium of pregnant and pseudopregnant rat following treatment with progesterone-receptor inhibitors including RU486 2) localization of COMT in macrophages of the corpus lutea and cervical lymph nodes of rat 3) activity and localization of COMT in the hamster kidney and in estrogen-induced carcinomas of hamster kidney 4) activity and localization of COMT in human endometrial and breast adenocarcinomas 5) the identification of berberine as additional products of the O-methylation 3'-hydroxycoclaurine 6) the identification of monoamine oxidase A and B as one of the irreversible binding sites of procaine isothiocyanate 7) the identification and tissue distribution of 2-fluorourocanic acid in mouse in vivo.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31102-22 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)

**Ion Channels, Receptors and Second Messengers in the Nervous System**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and

P.I.	J.W. Daly	Chief	LBC, NIDDK
Others:	B. Badio	IRTA	LBC, NIDDK
	W. Padgett	Biologist	LBC, NIDDK
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T. Yasumoto, Tohoku Univ., Sendai, Japan; R.A. Aronstam, Univ. GA, Augusta, GA; R.A. North, Vollum Institute, Portland, OR; F. Gusovsky, Eisai Res. Inst., Andover, MA; B. Nikodijevic, NICHD.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.8

PROFESSIONAL:

0.6

OTHER:

1.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Calcium, sodium, potassium, and magnesium ions after translocation through ion channels or by transport proteins can cause activation of release processes, contractile proteins, adenylate and guanylate cyclase, phosphodiesterases, protein kinases, phospholipases, ATPases and other enzymes. Receptors of various types and various toxins serve to modulate ion channels and generation of second messengers including cyclic nucleotides, diacylglycerides, arachidonic acid and phosphatidic acid. Modulatory interactions or "cross-talk" occurs both between the second messenger systems and with the ion transport systems. Maitotoxin, a marine polyether increases phospholipid, leading to inositol phosphate and diacylglyceride production. The primary site of action of maitotoxin appears to be a calcium channel similar in sensitivity to blockade by organic compounds to the so-called receptor-operated calcium (ROC) channels. The efficacy of blockade of maitotoxin-elicited calcium flux in fibroblasts by a variety of organic calcium channel blockers, including the antimitotic imidazoles (miconazole, econazole, etc) proposed as ROC channel blockers, does not correlate with anti-mitotic activity of the blockers in fibroblasts. The most potent blockers for maitotoxin-elicited calcium flux were certain of the imidazoles, R-verapamil and amiloride analogs containing a benzyl substituent on the amidine moiety. Pheochromocytoma cells appear to contain both an ATP and a UTP receptor, both of which lead to an increase in influx of calcium and generation of inositol phosphate. ATP and ATP analogues but not UTP triggers the following sequelae: A sustained elevation of intracellular calcium presumably through ROC channels. Release of norepinephrine. Activation of calcium-dependent potassium channels.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 DK 31104-25 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. M. Jerina	Section Chief	LBC, NIDDK
Others:	J. M. Sayer	Research Chemist	LBC, NIDDK
	H. Yagi	Visiting Scientist	LBC, NIDDK
	A. M. Cheh	Research Chemist	LBC, NIDDK
	N. T. Nashed	Special Expert	LBC, NIDDK
	M. K. Lakshman	Visiting Fellow	LBC, NIDDK
	B. Zajc	Visiting Fellow	LBC, NIDDK
	M. T. Haber	IRTA Fellow	LBC, NIDDK

COOPERATING UNITS (if any)

A. Conney, Rutgers U. (Newark, NJ); W. Levin and A. Wood, Roche Research Center (Nutley, NJ); D. R. Boyd, Queen's University of Belfast, N. Ireland; A. Dipple, FCRDC; H. Yeh, LAC, NIDDK, NIH; R. Loncharich, DCRT, NIH; A. Bax, LCP, NIDDK, NIH.

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

8

PROFESSIONAL:

7

OTHER:

1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The primary goal has been the elucidation of the structures of reactive metabolites responsible for the carcinogenic, cytotoxic and mutagenic activity of drugs, polycyclic aromatic hydrocarbons and other environmental chemicals. The approach taken consists of: i) study of the metabolism of the chemicals with liver microsomes and with purified cytochromes P450 and epoxide hydrolase, ii) synthesis of oxidative metabolites, iii) evaluation of the mutagenicity and tumorigenicity of the synthetic metabolites, iv) elucidation of the roles of the cytochrome P450 system and epoxide hydrolase in modulating the mutagenicity of these metabolites, v) determination of the rates and products of reactions of arene oxides and diol epoxides with biopolymers and model compounds, and vi) search for agents capable of preventing the tumorigenicity of reactive metabolites. Work during the past year has focused in two areas. i) Studies of the reaction mechanisms of K-region arene oxides have elucidated the role of the intrinsic chemical reactivity at each epoxide carbon. Conformational factors play a key role in the determination of rates and products of solvolytic cleavage and epoxide hydrolase-catalyzed hydrolysis. Our results also suggest that the transition state for nucleophilic attack of methoxide ion on the K-region arene oxides (a model for attack of water catalyzed by epoxide hydrolase) involves a partial positive charge on the carbon that undergoes nucleophilic substitution. ii) Synthetic studies have produced biologically significant oligonucleotides containing specific diol epoxide adducts. An adduct corresponding to trans opening of the (1R,2S)-diol (3S,4R)-epoxide of phenanthrene by the exocyclic amino group of deoxyadenosine (dA) was incorporated into a nonanucleotide comprising codons 60-62 of the human K-ras oncogene. In duplexes formed by this adducted oligonucleotide, substitution of deoxyguanosine for thymidine in the complementary strand opposite the modified dA had almost no effect on the melting temperature. This result suggests a possible role for such base-pair "mismatches" in mutations caused by diol epoxide adduct formation.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK31106-06 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanistic Enzymology of HIV Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	D.M. Jerina	Section Chief	LBC, NIDDK
Others:	J.M. Sayer	Research Chemist	LBC, NIDDK
	N.T. Nashed	Special Expert	LBC, NIDDK
	M.T. Haber	IRTA Fellow	LBC, NIDDK
	T.V.S. Rao	IRTA Fellow	LBC, NIDDK

COOPERATING UNITS (if any)

J.M. Louis and A.R. Kimmel, LCDB, NIDDK; K.D. Parriss, LMB, NIDDK

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.1

PROFESSIONAL:

3.0

OTHER:

0.1

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- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Enzymology, kinetics and synthetic, physical and analytical chemistry are used in mechanistic investigations of the reverse transcriptase and protease of human immunodeficiency virus type 1 (HIV-1), with the ultimate goal of developing specific inhibitors for these enzymes. i) In a previous report, we described the kinetics of proteolytic steps in the autoprocessing of the HIV-1 protease from a construct containing the protease sequence flanked by a 19-amino acid Pol sequence at the C-terminus and a 12-amino acid sequence from the trans frame peptide at the N-terminus, which is fused to the maltose binding protein of E. coli. We have now examined, by fluorescence spectroscopy, the renaturation of the fusion protein that initiates autoprocessing. Two processes were observed: an initial step (or steps) whose rate was too fast to measure using conventional mixing techniques, and a slower step that was attributed to cis/trans isomerization of peptide bonds involving the imino nitrogen of proline. The latter step is ca. 15 times faster than the initial proteolytic cleavage at the amino terminus of the protease sequence. This proteolytic step occurs both intramolecularly with the dimeric fusion protein alone, and intermolecularly in the presence of mature HIV-1 protease. Despite the low catalytic activity of the dimeric fusion protein, a fairly high concentration (1.4  $\mu$ M) of mature protease is required to give a rate that is competitive with intramolecular cleavage, as a result of the entropic advantage of intramolecularity. The 13.2-kDa protein intermediate that is formed by cleavage at the amino terminus of the protease sequence has been purified to >90% homogeneity. Preliminary results suggest that the dissociation constant for the dimeric form of this protein is much larger than that for either the full length fusion protein or the mature protease. ii) Kinetics of nonprocessive incorporation of 4-thio-thymidine into a DNA template-primer by the Klenow fragment of E. coli DNA polymerase I were measured spectrophotometrically at 335 nm. The rate constant for dissociation of the oligonucleotide product from the enzyme is ca. 6 times larger than the corresponding rate constant for its thymidine-containing analog.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK 31107-05

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mass Spectrometry of Drugs, Natural Products, Proteins, Oligonucleotides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L.K. Pannell Visiting Scientist LAC, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Oxidation Mechanisms

INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

2.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrefined type. Do not exceed the space provided.)

This project has been transferred to Z01-DK 58013-01 LAC.

2011

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## PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Adenosine Receptor Agonists and Antagonists

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and

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Others:	O. Nikodijevic	Visiting Associate	LBC, NIDDK
	R. Moni	Visiting Fellow	LBC, NIDDK
	Y. Shin	IRTA	LBC, NIDDK
	W. Padgett	Biologist	LBC, NIDDK
	Dan Shi	Special Volunteer	LBC, NIDDK

## COOPERATING UNITS (if any)

R. Olsson, U. So. Fla., Tampa, FL; C. Mueller, U. Tübingen, Germany; L.R. Brackett, Brown Univ. Providence, RI.

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Pharmacodynamics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL STAFF YEARS:

4.6

## PROFESSIONAL:

4.3

## OTHER:

0.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Adenosine regulates a wide range of physiological functions through interaction with at least two major classes of adenosine receptors. The A1 class of adenosine receptors is inhibitory to adenylate cyclase, while the A2 class is stimulatory to adenylate cyclase. Subclasses of adenosine receptors also occur. Some of these are inhibitory to calcium channels, some are stimulatory to potassium channels, some activate guanylate cyclase, some modulate phospholipid turn-over and some cause smooth muscle relaxation. Adenoregulin, a peptide isolated from an Amazonian hyliid frog, markedly stimulates binding of agonists to A<sub>1</sub>, A<sub>2</sub>, and 5-HT<sub>1A</sub> receptors. All of these receptors are coupled via G<sub>i</sub>-class proteins to adenylate cyclase. Adenoregulin at higher concentrations (>10  $\mu$ M) inhibits binding. The effects of adenoregulin appear related to alterations in receptor-G protein coupling, perhaps by enhancing such coupling. Another amphiphilic peptide mastoparan has similar effects on binding. Functional correlates with respect to effects of these peptides on adenylate cyclase have not been obtained, while stimulatory effects on calcium influx, transmitter release, and phosphoinositide are manifest for both peptides, but only at higher concentrations. Effects on phosphoinositide breakdown may relate to stimulation of calcium influx. In membranes and permeabilized cells both peptides are inhibitory to phosphoinositide breakdown. Structure activity relationships for adenosine analogs and xanthines have been further defined for A<sub>1</sub> receptors of fat and brain, for A<sub>2</sub> receptors of striatum, pheochromocytoma cells and human platelets, and for A<sub>2b</sub> receptors of fibroblasts. Certain analogs of caffeine are 4 to 5-fold more potent than caffeine in eliciting release of intracellular calcium. Chronic caffeine ingestion markedly alters density of many brain receptors and of calcium channels in parallel with changes in behavioral responses to adenosine analogs, xanthines, and cholinergic agents. High doses of caffeine elicit choreiform movements in mice and these are markedly reduced after chronic caffeine.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31109-04

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interaction between second messengers

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Fabian Gusovsky

Senior Staff Fellow

LBC, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Pharmacodynamics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Terminated



<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK. 31110-17 LBC
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Analogues of Thyrotropin-releasing Hormone</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Louis A. Cohen	Chief, Section on Biochem. Mech. NIDDK-LBC
Other:	Catherine Dong Tuan Tran	Visiting Fellow Stay-in-School NIDDK-LBC NIDDK-LBC
COOPERATING UNITS (if any) Dr. H. Kimoto, Nagoya, Japan; V. Labroo, Seattle, WA; S. Vonnhof, USUHS; A. Faden, Washington, DC		
LAB/BRANCH <u>Laboratory of Bioorganic Chemistry</u>		
SECTION <u>Section on Biochemical Mechanisms</u>		
INSTITUTE AND LOCATION <u>NIDDK, NIH, Bethesda, Maryland 20892</u>		
TOTAL MAN-YEARS:	1.8	PROFESSIONAL: 1.3 OTHER: .5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>           Analogs of the tripeptide hormone TRH, in which H-2 or H-4 of the imidazole ring has been replaced by a trifluoromethyl group, show pituitary and cardiovascular activities equal to, or better than, those of the natural peptide in the conscious rat. Thus, the loss of imidazole basicity and the introduction of a bulky substituent at either position seems to be unimportant in ligand recognition. Yet, the imidazole ring cannot be irrelevant to recognition since the 4-nitro analogue shows no pituitary activity but retains full cardiovascular activity. We have demonstrated the duality (or multiplicity) of TRH receptors in brain and pituitary. The further demonstration of separation of activities by our novel analogues offers an opportunity to attempt clinical removal of the set of activities governed by one receptor or another. (Trifluoromethyl)imidazoles show the very unique property of losing HF at mildly alkaline pH to form extremely electrophilic difluorodiazafulvenes. The generation of the latter species within a receptor's ligand-binding site may result in irreversible bond formation with a protein nucleophile in the binding site. At 37 °C and pH 8.5, the half-time for fulvene generation from 2-CF<sub>3</sub>-Im-TRH is ca. 30 hr. It is unreasonable, therefore, to search for irreversible binding to pituitary or brain tissue during the period of a normal incubation. Analysis of pK and reactivity data for a large number of additionally substituted (trifluoromethyl)imidazoles provided the basis for predicting appropriate extra substituents to give TRH analogues with half-lives ranging from 4 sec to 1.5 yr. We have chosen acetamido as the ideal substituent for our purposes and are now engaged in development of synthetic routes to 4-acetamido-2-trifluoromethyl-Im-TRH. Should this compound show the expected reactivity in binding irreversible to specific TRH receptors in tissue homogenates, it will then be administered to rats and followed by a dose of normal TRH. Strong diminution of either pituitary or CVS activity is expected.         </p> <p>           Similar approaches are being used to create selective irreversible inhibitors of histamine H1 and H2 receptors.         </p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01 DK 31111-23 LBC
PERIOD COVERED October 1, 1992 to September 30, 1993		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Stereopopulation Control in Drug Delivery and Enzyme Simulation		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Louis A. Cohen Chief, Section on Biochem. Mech.	NIDDK-LBC
Other:	Michael M. King Special Volunteer	GWU
COOPERATING UNITS (if any) Y. Ueno, Nagoya, Japan; W. Antkowiak, Poznan, Poland; Y. Takeuchi, Toyama, Japan; J. Flippen-Anderson, NRL, Washington, DC		
LAB/BRANCH Laboratory of Bioorganic Chemistry		
SECTION Section on Biochemical Mechanisms		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.2	
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>We have synthesized a large variety of test-tube models which simulate the enzyme-substrate complex by having the substrate frozen into a single, very favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (<u>stereopopulation control</u>). These compounds undergo intramolecular reactions at rates approaching those catalyzed by enzymes (but independently of any functional assistance). As part of our studies of practical applications of stereopopulation control, we have been exploring the use of various SPC-derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate transport from the gut to the circulatory system to the desired site of action by temporary masking of charge within the molecule, by improvement in lipophilicity and by regeneration based simply on local pH variation in ligand sites or on local concentrations of potent reducing agents. Recent studies have concentrated on o-nitrophenylpropionic acids as carriers. To date, these carriers have been coupled to GABA, to protected DOPA derivatives and to indoleamines. The nitro group is reduced enzymatically, and the resulting amine attacks an amide bond intramolecularly to release the drug. Rapid attack by the amine is ensured by placing a gem-dimethyl group on the adjacent carbon. Kinetic studies have shown that chemical reduction of the nitro group occurs at the same rate with or without the gem-dimethyl group, but that drug release is greatly enhanced by the action of the gem-dimethyl. Surprisingly, xanthine oxidase reduced the hindered nitro compound more readily than the unhindered. This result reveals that the enzyme must be able to reach a face of the nitro group for reduction, and not just an edge. Response to other reducing agents is now being explored with a view to using such prodrugs to deliver mustards and other anticancer agents to hypoxic cells.</p> <p>Similar model systems have been designed with conformationally frozen indole and phenolic rings, in order to simulate and study the tight charge transfer complexes achieved by tryptophan in proteins or by bioindoles in receptor binding</p>		



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31112-17 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Chemistry of Imidazoles and Bioimidazoles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen

Chief, Section on  
Biochem. Mech.

NIDDK-LBC

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

No activity during this period





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31113-17

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Halogenated Biogenic Amines in Biochemistry and Pharmacology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Kenneth L. Kirk	Chief, Section on Drug-Receptor Interactions	LBC, NIDDK
Other:	Bang-Hua Chen	Visiting Fellow	LBC, NIDDK
	Jun-ying Nie	Visiting Assoc.	LBC, NIDDK
	Mona Singh	IRTA Fellow	LBC, NIDDK
	Daniel Appella	Summer IRTA	LBC, NIDDK
	J. W. Daly	Chief	LBC, NIDDK
	C.R. Creveling	Research Chemist	LBC, NIDDK
	William Padgett	Chemist	LBC, NIDDK

COOPERATING UNITS (if any)

M. Channing, D. Kiesewetter (CC, Dept. of Nuclear Medicine), D.S. Goldstein (HE, NHLBI), R.S. Phillips (Univ. of Georgia).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Drug-Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.6

PROFESSIONAL:

3.2

OTHER:

0.4

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects      (b) Human tissues      (c) Neither  
(a1) Minors  
(a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Biogenic amines play key roles in neurotransmission, metabolism, and in control of various physiological processes. Using a variety of synthetic methodologies, including novel procedures developed by us, we have prepared a series of biogenic amines with fluorine substituted at various ring-positions. By virtue of its very small size and high electronegativity, fluorine is a very favorable replacement for hydrogen in these analogs. The biological properties and usefulness of these ring-fluorinated biogenic amines have proved to be extremely rewarding and continue to find applications in a multitude of studies, including research on the mechanisms of transport, storage, release, metabolism, and modes of action of these amines. Of particular significance was the discovery that 6-fluoronorepinephrine is a selective  $\alpha$ -adrenergic agonist and 2-fluoronorepinephrine is a selective  $\beta$ -adrenergic agonist. Mechanisms considered to explain these results include: 1) a direct effect of the C-F bond on agonist-receptor interaction or 2) an indirect effect of the C-F bond on the conformation of the ethanolamine side-chain. The results of testing of new analogs synthesized to probe these mechanisms indicate that electronic effects may be more important than conformational factors. Fluorinated analogs are useful mechanistic probes and biological tracers. The increased phenol acidities of fluorinated catecholamines proved to be an effective probe for details of the mechanisms of catechol methylation by the enzyme, catechol O-methyltransferase. We now have used the increased phenol acidities of fluorinated norepinephrines to study further the relationship between pH and adrenergic activity. [ $^{18}\text{F}$ ]-labeled 6-fluorodopamine, the biological precursor to 6-fluoronorepinephrine, has been found to be an excellent scanning agent for peripheral noradrenergic innervation. Research is in progress to develop central noradrenergic scanning agents based on this series.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 31114-11 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Significance of Ligand Tautomerism in Biorecognition

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen

Chief, Section on  
Biochem. Mech.

NIDDK-LBC

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

No activity during this period



DEPARTMENT OF HEALTH AND HUMAN SERVICES : PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31115-10 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Functionalized Congeners of Bioactive Compounds

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. A. Jacobson	Research Chemist	LBC, NIDDK
Others:			
	P. J. M. van Galen	Visiting Fellow	LBC, NIDDK
	B. Fischer	Visiting Fellow	LBC, NIDDK
	X.-D. Ji	Visiting Associate	LBC, NIDDK
	N. Melman	Special Volunteer	LBC, NIDDK

COOPERATING UNITS (if any)

J. Baumgold (GW University); G. Stiles (Duke University); Y. Karton (Israel Inst. Biological Research, Nes Ziona, Israel); E. Heilbronn (Univ. Stockholm); K. Harden (U.N.Carolina); G. Burnstock (U. College London)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Drug Receptor Interactions Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.7

PROFESSIONAL:

1.7

OTHER:

0

CHECK APPROPRIATE BOXES

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

Recent work in our laboratory has demonstrated that certain drugs may be attached to well-defined "carrier" molecules and still retain the ability to bind to the receptor site and effect biological activity. This synthetic strategy for the attachment of drugs to carriers is termed the "functionalized congener" approach. The "carrier" molecule may be many times larger than the parent drug; indeed there is practically no maximum size limitation for a fully potent analog. Unlike the prodrug approach or the immobilization of drugs for slow release, the "functionalized congener" approach is designed to produce analogs for which no metabolic cleavage step is necessary for activation. Moreover, the attachment of the drug to a "carrier" such as a peptide may result in enhanced affinity at an extracellular receptor site and an improvement in the pharmacological profile of the parent drug.

Purine derivatives containing attached chains were developed as functionalized congeners for adenosine receptors and for ATP receptors. Reporter groups such as fluorescent dyes have been covalently attached resulting in receptor probes of relatively high affinity. Sites for chain derivatization on the structures of telenzepine (useful drugs in treating stomach ulcers and as research tools for the brain), a selective muscarinic antagonists, have been located. In a series of amino alkyl derivatives, it was found that increasing the chain length enhances the potency of the derivative as a muscarinic antagonist. By incorporation of a phenyl isothiocyanate group, chemically reactive affinity labels for muscarinic receptors were developed. Other reporter groups included in the telenzepine series include biotin, p-aminophenylacetyl (for preparing radiotracers and photoaffinity labeling reagents), and fluorescent dyes fluorescein and tetramethylrhodamine (for locating the receptor sites microscopically and for binding assays that do not require the use of radioisotopes).



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31116-06 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Prosthetic Groups for Labeling of Functionalized Drugs and Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K.A. Jacobson

Research Chemist

LBC, NIDDK

COOPERATING UNITS (if any)

P. Skolnick (NIDDK); J. Baumgold (NIDDK); M. Channing (NM-CC); G. Stiles (Duke University)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Drug Receptor Interactions Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard/unreduced type. Do not exceed the space provided.)

The use of radioisotopes to label organic compounds for use in diagnostic nuclear medicine is well documented in the literature. It has been found that certain radiolabeled compounds will localize in the brain, heart, or in other target organs or tissues to a sufficient level to allow for imaging thereof. There has been increasing interest in finding compounds which will more effectively cross the blood-brain barrier, thus facilitating more efficacious imaging of the brain.

Binding sites for certain drugs in an animal or organ may be localized as a result of the synthesis of high specific activity radiolabeled analogs which have high affinity for that binding site. Prosthetic groups may be attached to a drug or other receptor ligand for the purpose of efficient and selective chemical capture of a particular radioisotope. Having developed functionalized congeners of theophylline and other drugs acting at adenosine receptors, we are now developing prosthetic groups for radioisotopes such as 18-F, 123-I, and 125-I, to be coupled to these functionalized drug molecules. The prosthetic groups contain amino or carboxylic groups which are to be condensed covalently to functionalized drugs to give conjugates of high affinity at a particular receptor, or drugs that bind the label irreversibly (trifunctional reagents). We have developed a radioiodinated derivative that contains an azido group for covalent reaction with A2-adenosine receptors.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31117-06 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Development of Drugs Acting at Adenosine Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. A. Jacobson	Research Chemist	LBC, NIDDK
Others:	X.-D. Ji	Visiting Associate	LBC, NIDDK
	P. J. M. van Galen	Visiting Fellow	LBC, NIDDK
	M. Maillard	Special Volunteer	LBC, NIDDK
	N. Melman	Special Volunteer	LBC, NIDDK
	D. von Lubitz	Special Volunteer	LBC, NIDDK
	C. Gallo-Rodriguez	Special Volunteer	LBC, NIDDK

COOPERATING UNITS (If any)

G. Stiles (Duke Univ.); R. T. Bartus (Cortex Pharmaceut.); K. Lee (Univ. Virginia); L. Belardinelli (U. Florida); R. A. Olsson (Univ. So. Florida); K. LaNoue (Univ. Penn.); A.P. Ijzerman (Ctr. for Bio-Pharmaceut.Sci., Leiden, The Netherlands).

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Drug Receptor Interactions Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.7

PROFESSIONAL:

1.7

OTHER:

0

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

The extracellular adenosine receptor has a modulatory role in the nervous, circulatory, endocrine, and immunological systems. The prospect of harnessing these effects specifically for therapeutic purposes is attractive.

We have developed research tools for the characterization of adenosine receptors in vitro and in vivo. We have synthesized new drug analogues and elucidated structure activity relationships at receptor subtypes. Derivatives of adenosine with chemical modifications at the N6 and C-2 positions of the purine ring act as selective adenosine agonists. A1-agonists are being explored as cerebroprotective agents. To enhance brain uptake, prodrug schemes are being examined. APEC, and A2 selective adenosine amine congener served as the basis for a photoaffinity labeling reagent that allowed the first determination of the molecular weight of the receptor. Functionalized congeners of xanthines act as potent adenosine antagonists and are being developed as radioactive tracers for adenosine receptors and as affinity labels. Peripherally selective adenosine agonists have been developed.

Since the two major subtypes of adenosine receptors have been cloned it has been possible to conduct molecular modeling of the receptor protein, based on sequence analyses and computerized energy minimizations. A hypothesis concerning the mode of binding to ligands to adenosine receptors has been derived. This hypothesis is consistent with pharmacological observations and site directed mutagenesis experiments, in which key histidyl residues have been replaced by other amino acids.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31118-04 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Bioindoles and Oxindoles as Medicinal and Diagnostic Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen Chief, Section on NIDDK-LBC  
Biochem. Mech.

Other: Yigal Fraenkel IRTA Fellow NIDDK-LBC

COOPERATING UNITS (if any) Peter Kador, NEI; J. Flippen-Anderson, NRL, Washington, DC;  
R. Labroc, Seattle, WA; P. A. Cohen, Vancouver, BC; Sanford Markey, NIMH

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.2

PROFESSIONAL:

1.2

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Interest in the physiological activities and potential clinical uses of the pineal hormone melatonin (N-acetyl-5-methoxytryptamine) has grown enormously in recent years. The hormone has been implicated in the regulation of phenomena as diverse as the sleep-wake cycle, depression, sexual urge, neuroimmunomodulation, mammary cancer, etc. We have mounted the first systematic program to develop agonists and antagonists for the hormone, and affinity labels for its multiple receptors. 2-Iodo- and 2-bromomelatonin can now be made by one-step syntheses and bind to the known receptor much more effectively than melatonin itself. Our new and greatly improved syntheses for these compounds will also facilitate preparation of labelled compounds for radioimmune assay. The superior binding implies that the receptor contains a large lipophilic hole, which may bind other 2-substituted melatonin analogues even more effectively. Synthetic methods are now being developed to obtain such analogues by radical alkylation procedures, which proved so effective in producing 2-alkylhistidines and histamines.

Studies have continued on the use of oxindoles as inhibitors of aldose reductase as a means to control diabetic retinopathy. We have now achieved inhibitory potencies (in vitro) equal to, or greater than, those of the best inhibitors generated by pharmaceutical industry, but have totally eliminated incorporation into the structures of strong allergy-inducing hydantoins. New work involves the development of prodrugs suitable for oral administration, based on the slow release of malonic acid structures from their esters by nonenzymatic hydrolysis.

Several years ago, we made a concerted effort to obtain 2-fluoroindoles by halogen exchange with 2-bromoindoles, but were unsuccessful. Direct fluorination by use of new fluorinating agents has now been found surprisingly successful and such compounds (2-fluoroserotonin, 2-fluoromelatonin) are anticipated to find application as affinity labels, analogues of peptide hormones and PET scanning reagents (since the introduction of radiolabel requires only one step).



## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31119-04 LBC

## PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Novel Amino Acids for Conformational and Stereochemical Constraints in Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen

Chief, Section on  
Biochem. Mech.

NIDDK-LBC

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Biochemical Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS.

PROFESSIONAL:

OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

No activity this year



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31120-04 LBC

PERIOD COVERED  
October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Fluorinated Analogues of Bioactive Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen

Chief, Section on  
Biochem. Mech.

NIDDK-LBC

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been temporarily discontinued





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 31121-03 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemistry and Biology of Novel Pyrimidine and Purine Nucleosides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen Chief, Section on NIDDK-LBC  
Biochem. Mech.

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Biochemical Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

No activity during this period



Z01 DK 31122-03 LBC

We have found 2-iodo-L-histidine to be a potent antimalarial agent against drug-resistant Plasmodium falciparum. However, the compound is effective in monkeys for only 24-48 hrs; we have shown that inactivation may be the result of nonenzymatic deiodination by any of the sulfhydryl compounds present in tissue or serum. The facts that 2-iodohistidine does not block protein synthesis in the parasite, and that the corresponding 2-chloro and 2-bromo compounds are inactive, led us to speculate that the 2-iodo compound operates by "plugging" one or more holes in the erythrocyte membrane and, thus, depriving the parasite of nonamino acid nutrients obtained by diffusion through such holes. Indeed, the diameter of the holes has been estimated at 0.7 nm, almost exactly the width of 2-iodohistidine. We have, therefore, explored the antimalarial activities of more metabolically stable derivatives of histidine which have ring substituents offering similar steric sizes. New general synthetic methods were devised to provide these series of compounds: 1-R-histidines, 2-R'-histidines, 1-R-2-R'-histidines (and the corresponding histamines), in which R need not equal R' and in which R and R' may be any saturated, unsaturated, cyclic, bicyclic, arylalkyl or heteroarylalkyl group, or any of their functionalized derivatives. Regiospecific alkylation at N-1 involves the use of cycloureidohistidine; despite the reduced nucleophilicity at N-1, alkylation occurs readily even with such hindered groups as cyclohexyl and t-butyl. Alkylation at C-2 involves the rarely used Minisci reaction, in which R'COOH is decarboxylated to R' radical by a catalytic amount of argentic ion (regenerated by a peroxy salt). In acidic media, the reaction is remarkably specific for alkylation at C-2 in good yield, providing the most efficient and least expensive method yet developed for the production of 2-alkylimidazoles in general, and 2-alkylhistidines or histamines in particular. In vitro screening of a random sampling of these compounds reveals modest inhibitory activity, but sufficient to support the concept of chemotherapy by membrane plugging, or "permeatherapy." Screening of a large number of these histidine or histamine analogs is in process. 191



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 32001-2 LBC

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Multifunctional Chemotherapeutic Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. Vithal Shetty Guest Researcher LBC, NIDDK

Others: K. L. Kirk Chief, Section on Drug Receptor Interactions LBC, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Drug Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.4

PROFESSIONAL:

0.4

OTHER:

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- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

A novel approach to the design of new antimicrobial agents has been developed. The overall purpose of the program is the rational design and synthesis of compounds that have combined antibacterail, anti-viral and anti-fungal activity. Specifically targeted in the rational design are enveloped viruses such as herpes viruses and HIV. These compounds have novel structural features which include a dimeric attachment of a known hydrophobic antimetabolite (for example, amino-adamantane analogues) through a very hydrophilic bridge. These structural features combine to provide the potential for a novel mechanism for attachment of the toxic moiety to microbial organisms. Thus, the hydrophilic portion of the molecule has a high affinity for glycoprotein components of the microbial cell wall. This affinity will deliver the toxic moieties to the cell surface, providing a mechanism for efficient activity. For example, the potential exists for inhibition of replication of such viruses as HIV and, through the attachment of the toxic molecule to the viral coat, a mechanism for killing the virus. Compounds in the series were found to have potent activity against gram positive and gram negative bacteria, fungi, yeast and enveloped viruses. Such combined activity potentially could be extremely useful in treatment of immunosuppressed patients.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 32002-1

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthesis and Biochemistry of Ascorbic Acid Analogues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kenneth L. Kirk Chief, Section on LBC, NIDDK  
Drug Receptor Interactions

Others: Arthur J. Crossman, Jr. IRTA FELLOW LBC, NIDDK

COOPERATING UNITS (if any)

Mark Levine, Richard Welch, Yaohui Wang (Nutrition Program, LCBG, NIDDK)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Drug-Receptor Interactions

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human subjects (b) Human tissues (c) Neither  
(a1) Minors  
(a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Ascorbic acid (vitamin C), a dietary requirement for human health, is an electron donor for several enzymatic actions, functions as an antioxidant, and is implicated in host defense mechanisms, endocrine function and the visual process (lens). Recent renewed interest in the biochemistry of ascorbic acid has been prompted by the realization that relatively little is known concerning the concentrations of the vitamin required for optimum functioning of these several roles. In the case of enzymatic reactions, optimal rate of a process is defined as that concentration that allows the reaction to reach Vmax without toxicity. As part of a program to determine these concentrations, in situ kinetic measurements have been carried out for certain vitamin C-linked reactions. In addition to examination of functional roles of vitamin C, recent characterization of efficient transport mechanisms that translocate vitamin C across cellular membranes has emphasized the importance of the vitamin to biological processes. Kinetic parameters of these transport mechanisms are also being determined.

The above work, carried out in the Nutrition Program, LCBG, NIDDK, has produced valuable information related to vitamin C biochemistry and metabolism, and has practical implications regarding dietary requirements. However, as in any biochemical study, structural analogues of the natural substrate would be extremely useful to further characterize substrate-macromolecule interactions, by functioning as inhibitors of transport processes, as enzyme inhibitors, and as potential agents for covalent labeling of the functional proteins involved in these processes. Unfortunately, relatively little synthetic work has been carried out on structural modifications of vitamin C. Examples of previous work include the preparation of 6-halo-6-deoxy- and 6-deoxy-ascorbic acid analogues, and 3- or 4-O-alkyl ethers of ascorbic acid. As part of a program to study the effects of structural modification on vitamin C function and to provide biochemical tools for vitamin C research, we have prepared the known 6-halo-6-deoxy analogues and have initiated kinetic measurements of transport inhibition of these analogues. In addition, we have developed reactions that should provide routes to new analogues that can function as affinity labels, radiotracers, and NMR probes.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 32003-1 LBC

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on Neurotransmitter Receptor Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others Zipora Pittel, Ph.D. Visiting Fellow LBC, NIDDK  
Klaus Bluml Special Volunteer LBC, NIDDK

COOPERATING UNITS (if any)

S. Gutkind, Ph.D. (LCDO, NIDR)

C. Felder, Ph.D. (LCB, NIMH)

LAB/BRANCH

Laboratory of Bioorganic Chemistry

SECTION

Section on Drug Receptor Interactions

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0

CHECK APPROPRIATE BOXES:

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

To elucidate the molecular basis underlying the function of G protein-coupled receptors, muscarinic acetylcholine receptors (m1-m5) were used as a model system. The molecular mechanisms involved in ligand binding, receptor activation, G protein coupling, and receptor assembly were studied by using a variety of different mutagenesis techniques.

Pharmacological studies with chimeric m2/m5 muscarinic receptors showed that the three-dimensional structure of muscarinic receptors (and most likely, other G protein-coupled receptors) resembles that of bacteriorhodopsin in that the seven transmembrane domains (TM I-VII) are arranged in a ring-like fashion such that TM I lies directly adjacent to TM VII.

Mutational analysis of the m3 muscarinic receptor led to the identification of several conserved Thr and Tyr residues (location: TM III, V, VI, or VII) which are critically involved in ACh binding and agonist-induced receptor activation. Systematic mutational modification of the N-terminal domain of the third cytoplasmic loop of the m3 receptor showed that a single amino acid (Tyr254; rat m3 receptor sequence), which is found in many other G protein-coupled receptors, is essential for the efficient activation of G proteins mediating stimulation of phosphatidylinositol hydrolysis.

Experiments designed to study the functional roles of amino acids that are highly conserved among all G protein-coupled receptors showed that four conserved Pro residues (location: TM IV, V, VI, and VII) play key roles in receptor expression, ligand binding and receptor function.

Coexpression studies with fragmented m2 and m3 receptors showed that muscarinic receptors behave in a fashion analogous to two-subunit receptors (one subunit containing TM I-V, and the other one, TM VI and VII). In addition, coexpression experiments with mutant m3 and chimeric adrenergic/muscarinic receptors showed that muscarinic receptors are able to interact with each other functionally at a molecular level.

The studies described above, together with biophysical and molecular modeling studies, should eventually lead to a detailed structural model of the ligand-receptor-G protein complex which should provide a rational basis for the development of novel muscarinic drugs.



The Laboratory of Molecular Biology has as its principal goal the understanding of biological processes at the molecular level. The research program involves a broad range of experimental approaches to problems in molecular genetics, the regulation of gene expression in prokaryotes and eukaryotes, and the structures of nucleic acids and proteins. Current research includes studies of the organization of DNA and proteins within the eukaryotic nucleus, studies of the molecular mechanisms for establishing and maintaining stable states of gene expression during embryonic development, studies of site-specific recombination mechanisms in a variety of systems including retroviruses, bacteriophage, and the genes of the immune system, and studies of DNA supercoiling and its regulatory effects as well as studies of the molecular mechanisms used by enzymes responsible for supercoiling. More direct structural research includes studies on the chemistry, structures and interactions of polynucleotides, calorimetric studies of nucleic acids and proteins, investigations of molecular crowding in biological systems, and a large number of crystallographic studies of the structures of enzymes, viral proteins, and immunoglobulins. Significant advances have been made during the past year in all of these areas.

### Chromatin Structure and Function

We have continued our studies of chromatin structure in the neighborhood of expressed genes. The globin gene family in chicken erythroid cells serves as a model system in which it is possible to study the mechanisms associated with regulation of the individual members of the family during erythroid development. We have extended our studies of stage-specific erythroid expression in the  $\alpha$ -globin gene family to the chicken embryonic  $\alpha^H$ -globin gene, and shown that changes in the concentrations of three trans-acting factors that interact and that vary in concentration during development may account for much of the stage-specific expression. We have also extended studies of the structure and function of the general erythroid-specific factor GATA-1. We have continued investigation of the effects of chromatin structure on gene expression.

We have investigated the structure and genesis of locus control regions (LCRs) in the human and chicken  $\beta$ -globin loci. We have also identified a DNA sequence element far upstream in the chicken  $\beta$ -globin locus that can serve as an insulator to block the action of an LCR/enhancer on a promoter. The insulating element functions both in human erythroid cell lines and in *Drosophila*, and may be part of a complex marking the boundary between chromatin domains. Work also continues on the effects of supercoiling on chromatin structure, and on the mechanism by which RNA polymerase transcribes through chromatin. We find that transcription through nucleosome cores involves an intramolecular transfer in which the core is displaced backward on the DNA without leaving the template.

### Studies on the Mechanism of Genetic Recombination

The major objective of this project is to uncover the molecular mechanisms of a variety of genetic rearrangements. The transposition reaction of bacteriophage Mu is studied as a model system.

Critical steps in Mu transposition are a pair of DNA cleavages and strand transfers which generate a branched DNA intermediate. Efficient formation of this intermediate requires the phage-encoded MuA and MuB proteins and the *E. coli*-encoded HU and IHF proteins, ATP and Mg<sup>++</sup>. The MuA protein interacts with two distinct types of DNA sequences, one type is at the ends of the Mu genome while the other lies internally at the Mu operator. Interactions involving multiple MuA molecules, accessory protein factors and sequences on the donor DNA lead to formation of a stable protein-DNA complex in which the two Mu ends are synapsed by a tetramer of MuA. Next, a pair of single strand cuts are made to expose the 3' ends of the Mu sequence. This cleaved donor DNA remains tightly associated with the MuA tetramer and this complex efficiently captures a "target" DNA molecule provided it is bound by MuB protein. A staggered cut is introduced into the target DNA and the two 5' ends are joined to the 3' ends of the Mu end sequences in a concerted reaction. Evidence has been obtained that this



reaction takes place by a one-step transesterification mechanism.

The assembly process and the functional organization of the MuA tetramer-Mu DNA complex have been studied by making use of a variety of mutant MuA proteins with missing functional domains.

The MuB protein, an ATPase, selectively stimulates utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The MuB protein dissociates preferentially from DNA molecules bound by MuA protein in a process that depends on ATP hydrolysis, preventing self-destruction of Mu DNA by transposition into the Mu sequence.

#### Studies of the Mechanism of Retroviral DNA Integration

A critical step in the retroviral replication cycle is integration of a DNA copy of the viral genome into a chromosome of an infected cell. The objectives of this project are to understand the detailed molecular mechanism of this step in the retroviral replication cycle. In vitro assay systems developed in this laboratory have enabled us to previously determine many key features of the reaction mechanism. Our work continues to focus on the biochemical activities of the HIV-1 integrase protein.

The HIV-encoded integrase protein catalyzes both the 3' processing reaction that cleaves two nucleotides from the 3' ends of the viral DNA prior to integration, and also the subsequent DNA strand transfer step that inserts the viral DNA ends into a target DNA; integrase can also promote an apparent reversal of the DNA strand transfer reaction termed disintegration.

We have constructed an extensive set of point mutations in the HIV integrase gene and purified the mutant proteins. Each protein has been assayed for 3' processing, DNA strand transfer, and "disintegration" activities. The results demonstrate that the central core region of integrase is both necessary and sufficient for the disintegration reaction and therefore contains the active site for polynucleotidyl transfer. However, additional functions supplied by the N-terminal and C-terminal regions of integrase are necessary for the 3' processing and DNA strand transfer activities.

Mutant integrase proteins that have little or no activity when assayed alone complement to restore near wild type levels of activity when assayed as certain pairwise mixtures. This result demonstrates that integrase functions as a multimer in both the 3' processing and DNA strand transfer reactions. Inspection of the pairs of mutant proteins that are able to complement reveals that, within the active multimer, the N-terminal domain of one monomer functions together with the catalytic domain of another monomer.

Some features of the integration process are not fully reproduced in simple reaction systems containing only integrase and DNA substrate. These aspects of the integration process are being investigated by studying integration complexes isolated from cells infected with Moloney murine leukemia virus.

#### Studies of Immunoglobulin Gene Rearrangement

Our earlier work on antigen receptor gene rearrangement (V(D)J recombination) described one type of broken DNA molecules that were plausible intermediates in this process. These molecules, seen at the T cell receptor  $\delta$  locus (TCR), had only the free end containing the recombination signal sequence.

We have now also been able to find broken molecules with the end of the coding sequence, but only in DNA from mice with the scid mutation, which interferes with the joining of these ends. The coding ends are found to have an abnormal structure: the two DNA strands are linked to each other in a hairpin configuration. The presence of hairpins offers a good explanation for the observation, in immunoglobulin and T cell receptor coding junctions, of self-complementary sequences formed during recombination. If a hairpin structure is nicked off-center, a self-complementary



insertion will naturally result.

These results strengthen the evidence for a breakage-reunion model of V(D)J recombination, but also indicate that the process must be different from earlier examples of this type.

### Studies of Functions Involved in Genetic Recombination

A novel topoisomerase (topoisomerase V) from a thermophilic archaeobacterium has been shown to have properties similar to eukaryotic topoisomerases and quite distinct from other bacterial enzymes of this class. It relaxes either negatively or positively supercoiled DNA in a reaction not dependent on a divalent metal ion, attaches to the 3' end of the DNA chain, and shows a striking cross-reactivity with antibody against human topoisomerase I. An evolutionary relationship between archaeobacteria and eukaryotes has often been discussed; the properties of this enzyme support the idea.

In further studies of DNA gyrase, mutations of the ATP binding site have been used to show that both B subunits in the active  $A_2B_2$  complex must be capable of hydrolyzing ATP in order for the enzyme to catalyze DNA supercoiling.

Transcription from *E. coli* promoters that are stimulated by DNA relaxation is also able to read through termination signals some distance away, and this readthrough is further increased by DNA relaxation. This coupling between events at separated sites is evidence for the use of other factors beside RNA polymerase. An assay for such factors has been devised and is now being used in a purification scheme.

### Nonheritable Antibiotic Resistance

We previously reported that growth in salicylate increases the resistance of a number of Gram-negative bacteria to a variety of clinically important antibiotics. We have traced part of this effect in *Escherichia coli* to a cascade of molecular events initiated by induction of transcription of the *marRAB* (multiple antibiotic resistance) operon. One consequence of *mar* induction is increased transcription of *micF* whose mRNA is partly anti-sense to the mRNA of *ompF*. This results in a severe reduction in the translation of *ompF* mRNA into OmpF, a major outer membrane porin. The absence of OmpF reduces the permeation of the outer membrane by antibiotics. Thus, treatment with salicylate induces the same profile of antibiotic resistance that is found in rare constitutive *mar* mutants.

In addition to the effects on *micF* and *ompF*, induction of *mar* increases the expression of at least 5 other genes including Mn-superoxide dismutase and glucose-6-phosphate dehydrogenase. We have now identified *inaA*, a weak acid inducible gene, as being regulated by *mar*. *mar* constitutive (antibiotic resistant) mutants express *inaA* constitutively. Furthermore, *marA/B* is necessary for the inducibility of *inaA*, but not its basal level expression. Basal level expression of *inaA* requires the presence of other sequences mapped to within 39 kb of *mar*.

Substances that induce *mar* and *inaA* include weak acids (benzoate, acetylsalicylate, acetaminophen); uncouplers (2,4-dinitrophenol), antibiotics (chloramphenicol, tetracycline); and superoxide generators (paraquat, menadione). The nature of the sensor(s) involved in monitoring these seemingly diverse effectors is not known. Compounds that interfere with the sensors could reduce bacterial defense against antibiotics.

### Molecular Basis of Antibiotic Resistance

Mu-lac insertion mutants of *E. coli* with altered expression in the presence of salicyl alcohol or that fail to exhibit altered antibiotic sensitivity when exposed to salicyl alcohol have been isolated. Five antibiotic-sensitive mutants were selected on the basis that their resistance to nalidixic acid increased only minimally when grown in the presence of salicyl alcohol. Two of these mutants were also hyper-sensitive to nalidixic acid in the presence of sodium salicylate.





The failure of salicyl alcohol or salicylates to induce antibiotic resistance was drug-specific in four of the five mutants. In one, however, salicyl alcohol failed to induce resistance to ampicillin as well as to nalidixic acid. This mutant responded normally to both antibiotics when grown in sodium salicylate. The mutants have been roughly mapped by Hfr crosses and are currently being sequenced.

Over 100 mutants that fail to express  $\beta$ -galactosidase in the presence of salicyl alcohol have been isolated and are being examined. Preliminary data suggests that the genes mutated in this class are principally expressed in mid-log phase and not in early log phase or at saturation density. We are tentatively referring to these genes as mid-life crisis genes. No mutant was found that increased the expression of  $\beta$ -galactosidase in the presence of salicyl alcohol.

#### Thermal Measurements of Biomolecular Systems

On account of renewed interest in triple-stranded nucleic acid complexes we are carrying out a comprehensive thermodynamic study of helix formation among DNA homopolymers containing adenine, thymine and uracil. We have found that dissociation of a third strand of poly(dT) vs. that of poly(dU) from the same poly(dA).poly(dT) double helix over the range of 35°C to 75°C is characterized by a 400 cal/mol greater enthalpy change that is offset by a 1 cal/deg/mol entropy change. The heat capacity change in the reaction was found to be the same for the two different polymer systems. Since the heat capacity change is the hallmark characteristic of the hydrophobic effect, this result indicates that there is little change in solvent exposure of the thymine methyl groups upon dissociation of the third polypyrimidine strand. Since the structures of the two complexes are very likely identical, these experiments establish the magnitude of the energy changes in a well defined comparison between poly(dT) and poly(dU).

#### Influences of Macromolecular Crowding on Biochemical Systems

The high concentration of macromolecules within cells can result in large excluded volume effects. We have studied crowding effects under conditions that approach more closely to such cellular conditions by measuring reactions in the presence of very concentrated extracts from cells of *Escherichia coli*. We find the rate of a test reaction, the cohesion of complementary sequences of lambda DNA, to be accelerated by one or two orders of magnitude under such conditions.

The increased reaction rate is correlated with condensation of the DNA. Condensation requires a DNA-binding protein fraction from the extracts. The amount of DNA-binding proteins causing these results can be decreased greater than 10-fold by concomitantly supplying a background of a purified polymer such as PEG 8000 which provides a moderate level of crowding. We suggest that both cytoplasmic crowding effects and DNA-binding proteins are required to cause the massive condensation of DNA into nucleoids in bacteria, a phenomenon which has remained a poorly understood characteristic of prokaryotes.

A review of macromolecular crowding effects on genome structure and expression is being written for *Biochimica Biophysica Acta*. A more general review on macromolecular crowding in collaboration with Allen Minton has been completed.

#### ned crystals of DNA oligonucleotide helices which give fiber type X-ray Chemical and Structural Investigations of Nucleic Acids and Related Molecules

We have developed a detailed molecular model for the DNA triple helix dT dA dT, in which the helix has three symmetry elements: a pseudodyad relating the Watson-Crick strands, an exact dyad relating the two T strands, and a pseudorotational symmetry relating the Hoogsteen A and T strands. The structure is constrained to a small region of conformation space and has little flexibility. We have obtained crystals of DNA oligonucleotide helices which give fibre type diffraction patterns. These patterns support our model but indicate that the molecules are ordered only in the axial direction since the usual crystal diffraction spots are not seen.

We have obtained and characterized for the first time two-stranded DNA helices with



Hoogsteen base pairing. The structural constraints of the duplex are the same as those encountered in modeling the triple helix, of which the Hoogsteen duplex is a component, and serve to determine the structures of both helices. Nearly identical carbonyl vibration spectra of the Hoogsteen duplex and the triple helix (both quite different from that of the Watson-Crick duplex) are consistent with the dyad symmetry relating the two pyrimidine strands.

In reexamining the interaction of poly dT and poly dA we have completed the phase diagram and observed the disproportionation reaction  $2 A T \rightleftharpoons A T_2 + A$ , not previously reported in this system. Both the  $2 \rightarrow 3$  and  $3 \rightarrow 2$  transitions have unusually high salt independence, indicating that the DNA triple helix binds more  $Na^+$  than RNA triplexes, possibly because of closer proximity of the phosphates in the B form DNA helices.

#### Aids Related Proteins: Structure and Function

Several N- and C-terminal truncated forms of the HIV1 integration protein have been cloned, purified and subjected to crystallization procedures. Two forms consisting of amino acids 50-212 and 213-288 have dramatically improved solubility properties compared to the full length protein.

Solutions have been prepared consisting of an Fab of a monoclonal antibody with the full length HIV1 integration protein and are being subjected to crystallization procedures.

#### Three-Dimensional Structures of Cytokines, Receptors and Immune System Proteins

1) The crystal structure of the multifunctional cytokine, Transforming Growth Factor-beta (TGF-beta 2), has now been refined to 1.8A resolution. The molecule is a homodimer, with each subunit having an unusual open fold with a preserved disulfide-rich core. Comparison with other members of the TGF-beta family and with the members of the superfamily such as activins and inhibins indicate that they probably adopt very similar structures. The folding topology is also similar to that adopted by Nerve Growth Factor (NGF), and of Platelet Derived Growth Factor (PDGF), although the mode of dimer formation is quite different for all three proteins. It is suggested that these cysteine rich cytokines may have evolved with different dimer structures in order to diversify the functional role for this subunit fold.

2) As part of an investigation of the structural basis of antibody specificity, the crystal structure of the complex of the MAB HyHEL-5 with chicken lysozyme has been refined. Also, the structure of the complex of this antibody with a mutant lysozyme with 10,000 times weaker binding has been determined. In the mutant an arginine present in the wild type has been replaced with a lysine (R68K). A comparison of the mutant and the wild type has been made in order to explain the large effect of this conservative mutation on the affinity.

#### Enzyme Structure

The multi-enzyme complex, tryptophan synthase from *Salmonella typhimurium*, has been further analyzed by X-ray diffraction. High resolution data have been collected for two derivatives of a mutant, K87T of the beta subunit, that approximate enzyme catalyzed intermediates. These structures have been refined, to provide new information about the disposition of residues and their interaction with the intermediates of the beta reaction. In addition another mutant,  $\alpha D60E$ , has been analyzed and data have been collected for two inhibitor complexes of the  $\beta K87T$  mutant to examine the effect on flexible regions of the  $\alpha$  active site. The comparison of these many refined structures will provide insight into the mechanism of action of both subunits and the molecular basis of their interaction.

#### Structural Studies of Molecular Recognition

(1) A molecular replacement solution of the crystal structure of unliganded Fab of HyHEL-10 has been obtained and is being refined.

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- (2) The molecular replacement analysis of the crystal structure of the Fab of CC49, a murine monoclonal antibody against solid adenocarcinoma, is being pursued.
- (3) Crystals of a single-chain construct containing the  $V_L$  and  $V_H$  of CC49 fused to human IgG1  $C_H2$  and  $C_H3$  have been obtained.
- (4) Crystallographic studies on various other proteins have been initiated.
- (5) Models of the extracellular portions of the human and murine low-affinity IgE receptors (CD23) have been built.
- (6) The binding of various viral and self peptides to the murine class I MHC antigen, H-2D<sup>d</sup>, has been modelled.

#### Study of the Potential Use of Catalytic Antibodies Against AIDS

Two peptidic transition-state analogs, corresponding to segments in the gp120 sequence, have been synthesized and used as immunogens for the production of murine monoclonal antibodies. Studies using supernatants from clones secreting antibody, incubated for varying lengths of time with transition-state analogs and native peptides, suggest that cleavage of the latter does take place.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 DK 33000-27

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Functions Involved in Genetic Recombination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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LMB/NIDDK

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Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.2

PROFESSIONAL:

3.2

OTHER:

CHECK APPROPRIATE BOX(ES)

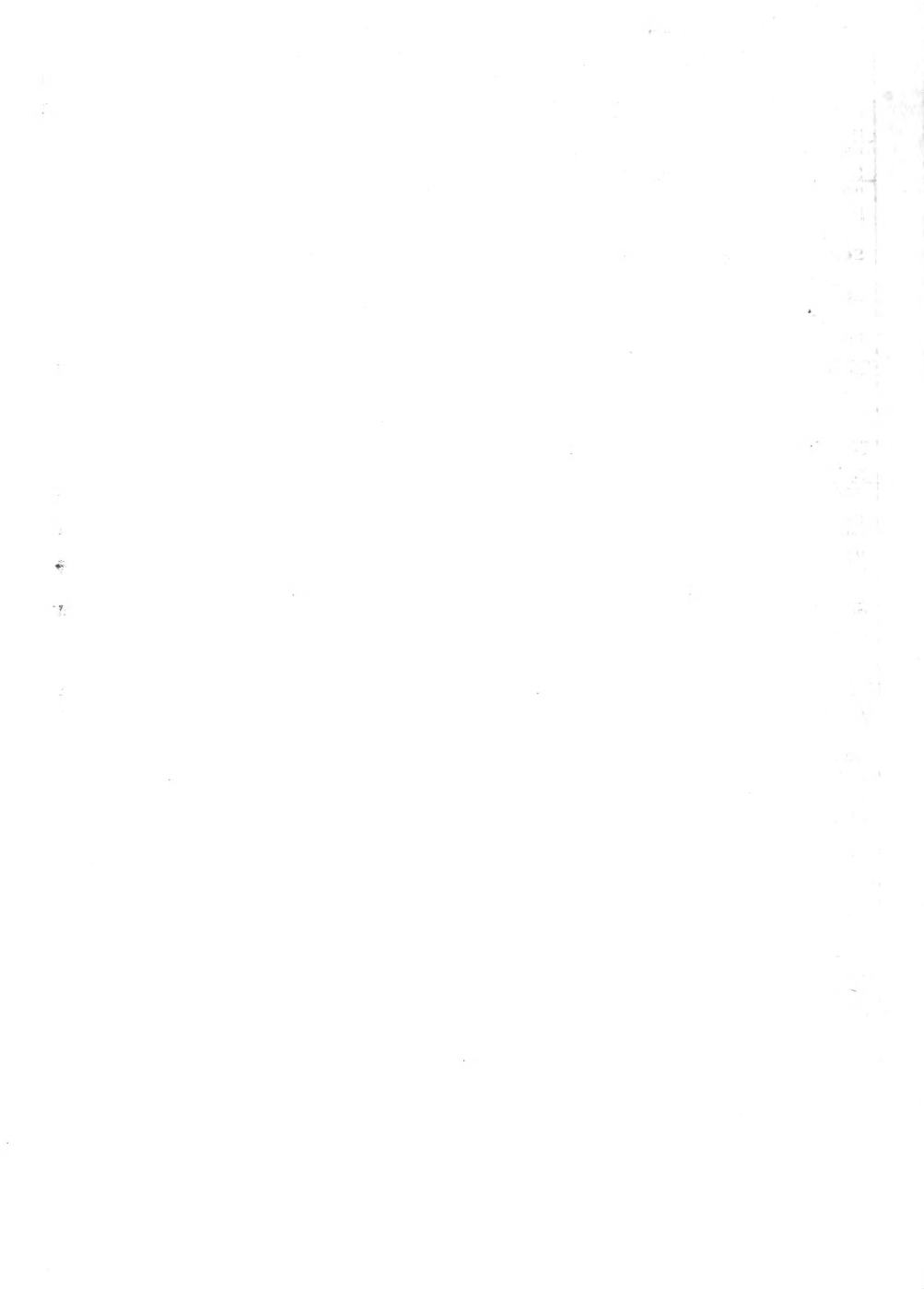
- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

A novel topoisomerase (topoisomerase V) from a thermophilic archaeobacterium has been shown to have properties similar to eukaryotic topoisomerases and quite distinct from other bacterial enzymes of this class. It relaxes either negatively or positively supercoiled DNA in a reaction not dependent on a divalent metal ion, attaches to the 3' end of the DNA chain, and shows a striking cross-reactivity with antibody against human topoisomerase I. An evolutionary relationship between archaeobacteria and eukaryotes has often been discussed; the properties of this enzyme support the idea.

In further studies of DNA gyrase, mutations of the ATP binding site have been used to show that both B subunits in the active  $A_2B_2$  complex must be capable of hydrolyzing ATP in order for the enzyme to catalyze DNA supercoiling.

Transcription from E. coli promoters that are stimulated by DNA relaxation is also able to read through termination signals some distance away, and this readthrough is further increased by DNA relaxation. This coupling between events at separated sites is evidence for the use of other factors beside RNA polymerase. An assay for such factors has been devised and is now being used in a purification scheme.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

701 DK 33001-9

PERIOD COVERED

**October 1, 1992 to September 30, 1993**

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Studies of Immunoglobulin Gene Rearrangement**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

**Martin Gellert, Ph.D.** Chief, Section on Metabolic Enzymes  
**LMB/NIDDK**

**Joanne Hesse, Ph.D.** Research Chemist

**LMB/NIDDK**

**Fraser McBlane, Ph.D.** Visiting Fellow

**LMB/NIDDK**

**Joseph Menetski, Ph.D.** Guest Researcher

COOPERATING UNITS (if any)

LAB/BRANCH

**Laboratory of Molecular Biology, DIR/NIDDK**

SECTION

**Section on Metabolic Enzymes**

INSTITUTE AND LOCATION

**NIDDK, NIH, Bethesda, Maryland 20892**

TOTAL STAFF YEARS:

**5.8**

PROFESSIONAL:

**5.8**

OTHER:

CHECK APPROPRIATE BOX(ES)

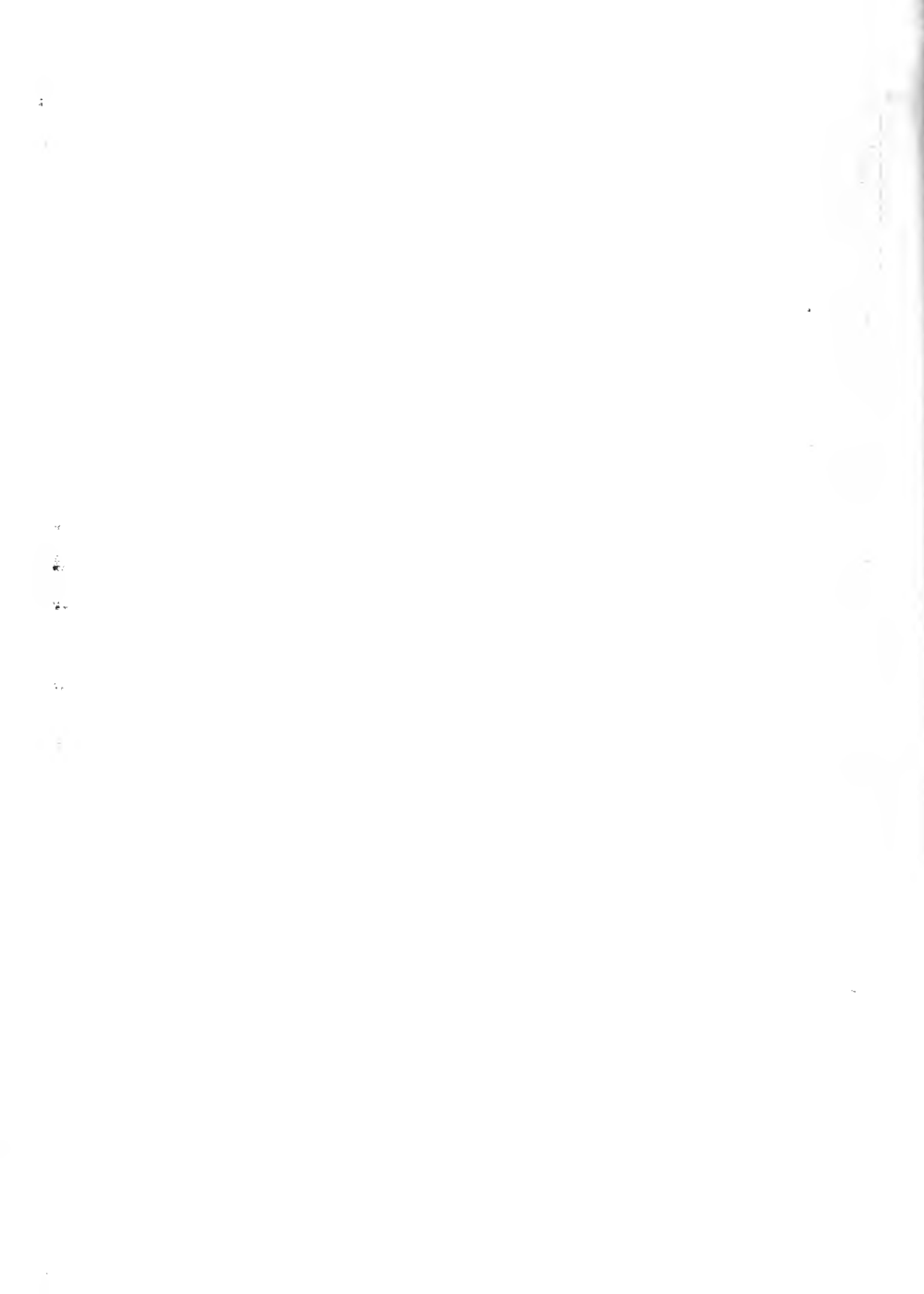
- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Our earlier work on antigen receptor gene rearrangement (V(D)J recombination) described one type of broken DNA molecules that were plausible intermediates in this process. These molecules, seen at the T cell receptor  $\delta$  locus (TCR), had only the free end containing the recombination signal sequence.

We have now also been able to find broken molecules with the end of the coding sequence, but only in DNA from mice with the scid mutation, which interferes with the joining of these ends. The coding ends are found to have an abnormal structure: the two DNA strands are linked to each other in a hairpin configuration. The presence of hairpins offers a good explanation for the observation, in immunoglobulin and T cell receptor coding junctions, of self-complementary sequences formed during recombination. If a hairpin structure is nicked off-center, a self-complementary insertion will naturally result.

These results strengthen the evidence for a breakage-reunion model of V(D)J recombination, but also indicate that the process must be different from earlier examples of this type.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 33006-15

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Mechanism of Genetic Recombination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Kiyoshi Mizuuchi	Chief, Section on Genetic Mechanisms	LMB/NIDDK
M. Mizuuchi	Visiting Associate	LMB/NIDDK
H. Savilahti	Visiting Fellow	LMB/NIDDK
P. Rice	IRTA Fellow	LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology, DIR/LMB

SECTION

Section on Genetic Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.5

PROFESSIONAL:

3.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

The major objective of this project is to uncover the molecular mechanisms of a variety of genetic rearrangements. The transposition reaction of bacteriophage Mu is studied as a model system.

Critical steps in Mu transposition are a pair of DNA cleavages and strand transfers which generate a branched DNA intermediate. Efficient formation of this intermediate requires the phage-encoded MuA and MuB proteins and the E. coli-encoded HU and IHF proteins, ATP and Mg<sup>++</sup>. The MuA protein interacts with two distinct types of DNA sequences, one type is at the ends of the Mu genome while the other lies internally at the Mu operator. Interactions involving multiple MuA molecules, accessory protein factors and sequences on the donor DNA lead to formation of a stable protein-DNA complex in which the two Mu ends are synapsed by a tetramer of MuA. Next, a pair of single strand cuts are made to expose the 3' ends of the Mu sequence. This cleaved donor DNA remains tightly associated with the MuA tetramer and this complex efficiently captures a "target" DNA molecule provided it is bound by MuB protein. A staggered cut is introduced into the target DNA and the two 5' ends are joined to the 3' ends of the Mu end sequences in a concerted reaction. Evidence has been obtained that this reaction takes place by one-step transesterification mechanism.

The assembly process and the functional organization of the MuA tetramer-Mu DNA complex have been studied by making use of a variety of mutant MuA proteins with missing functional domains.

The MuB protein, an ATPase, selectively stimulates utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The MuB protein dissociates preferentially from DNA molecules bound by MuA protein in a process that depends on ATP hydrolysis, preventing self-destruction of Mu DNA by transposition into the Mu sequence.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01DK 34001-28

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Gary Felsenfeld, Ph.D., Chief, Section on Physical Chemistry LMB/NIDDK  
David Clark, V. Assoc. LMB/NIDDK Jay Chung, Comm. Off. LMB/NIDDK  
Hannah Gould, Expert LMB/NIDDK Mark Minie, Staff Fel. LMB/NIDDK  
Cecelia Trainor, Staff Fel. LMB/NIDDK Henryk Eisenberg, V.Sci.LMB/NIDDK  
Rodolfo Ghirlando, V. Fel. LMB/NIDDK Vasily Studitsky, V.As LMB/NIDDK  
Joseph Grasso, Spec.Vol. LMB/NIDDK Emery Bresnick, Staff Fel LMB/NIDDK  
Joan Boyes, V. Fel. LMB/NIDDK Michael Pikaart, IRTA LMB/NIDDK  
Thomas Vandergon, Staff Fel LMB/NIDDK

COOPERATING UNITS (if any)

Laboratory of Chemical Physics, NIDDK (J. Omichinski, G.M. Clore, A. Gronenborn); Dept. of Biophysics, King's College (Robert Hannon); NICHD, NIH (Heiner Westphal); Division of Cellular and Gene Therapy,

LAB/BRANCH

Laboratory of Molecular Biology, DIR/NIDDK

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

PROFESSIONAL:

OTHER:

11.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unmodified type. Do not exceed the space provided.)

We have continued our studies of chromatin structure in the neighborhood of expressed genes. The globin gene family in chicken erythroid cells serves as a model system in which it is possible to study the mechanisms associated with regulation of the individual members of the family during erythroid development. We have extended our studies of stage-specific erythroid expression in the  $\alpha$ -globin gene family to the chicken embryonic  $\alpha^*$ -globin gene, and shown that changes in the concentrations of three trans-acting factors that interact and that vary in concentration during development may account for much of the stage-specific expression. We have also extended studies of the structure and function of the general erythroid-specific factor GATA-1. We have continued investigation of the effects of chromatin structure on gene expression. We have investigated the structure and genesis of locus control regions (LCRs) in the human and chicken  $\beta$ -globin loci. We have also identified a DNA sequence element far upstream in the chicken  $\beta$ -globin locus that can serve as an insulator to block the action of an LCR/enhancer on a promoter. The insulating element functions both in human erythroid cell lines and in Drosophila, and may be part of a complex marking the boundary between chromatin domains. Work also continues on the effects of supercoiling on chromatin structure, and on the mechanism by which RNA polymerase transcribes through chromatin. We find that transcription through nucleosome cores involves an intramolecular transfer in which the core is displaced backward on the DNA without leaving the template.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 DK 34002-28 LMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Enzyme Structure**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: David R. Davies, Chief, Sect. on Molecular Structure LMB/NIDDK

Others: Kevin Parris, Staff Fellow LMB/NIDDK  
 Gerson H. Cohen, Research Chemist LMB/NIDDK  
 Mi Li, Visiting Associate LMB/NIDDK  
 C. Craig Hyde, Special Volunteer LMB/NIDDK

COOPERATING UNITS (if any)

Edith Miles, NIDDK

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.8

PROFESSIONAL:

1.8

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unenlarged type. Do not exceed the space provided.)

The multi-enzyme complex, tryptophan synthase from *Salmonella typhimurium*, has been further analyzed by X-ray diffraction. High resolution data have been collected for two derivatives of a mutant, K87T, of the beta subunit that approximate enzyme catalyzed intermediates. These structures have been refined, to provide new information about the disposition of residues and their interaction with the intermediates of the beta reaction. In addition another mutant,  $\alpha$ D60E, has been analyzed and data have been collected for two inhibitor complexes of the BK87T mutant to examine the effect on flexible regions of the  $\alpha$  active site.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 DK 34003-25 LMB

PERIOD COVERED

**October 1, 1992 to September 30, 1993**

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Three-Dimensional Structures of Cytokines Receptors and Immune System Proteins**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

**P.I.: David R. Davies, Chief, Sect. on Molecular Structure LMB/NIDDK**

<b>Others:</b>	<b>Susan Chacko, Visiting Fellow</b>	<b>LMB/NIDDK</b>
	<b>Enid W. Silvertown, Research Chemist</b>	<b>LMB/NIDDK</b>
	<b>Sun Daopin, Visiting Scientist</b>	<b>LMB/NIDDK</b>
	<b>Gerson H. Cohen, Research Chemist</b>	<b>LMB/NIDDK</b>

COOPERATING UNITS (if any)

**Karl Piez, Special Volunteer, Section on Molec. Structure LMB/NIDDK**

LAB/BRANCH

**Laboratory of Molecular Biology/NIDDK**

SECTION

**Section on Molecular Structure**

INSTITUTE AND LOCATION

**NIDDK, NIH, Bethesda, Maryland 20892**

TOTAL STAFF YEARS:

**3.7**

PROFESSIONAL:

**3.7**

OTHER:

**0**

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unenriched type. Do not exceed the space provided.)

- 1) The crystal structure of the multifunctional cytokine, Transforming Growth Factor-beta (TGF-beta 2), has now been refined to 1.8A resolution. The molecule is a homodimer, with each subunit having an unusual open fold with a preserved disulfide-rich core. Comparison with other members of the TGF-beta family and with the members of the superfamily such as activins and inhibins indicate that they probably adopt very similar structures. The folding topology is also similar to that adopted by Nerve Growth Factor (NGF), and of Platelet Derived Growth Factor (PDGF), although the mode of dimer formation is quite different for all three proteins.
- 2) As part of an investigation of the structural basis of antibody specificity, the crystal structure of the complex of the MAB HyHEL-5 with chicken lysozyme has been refined. Also, the structure of the complex of this antibody with a mutant lysozyme with 10,000 times weaker binding has been determined. In the mutant an arginine present in the wild type has been replaced by a lysine (R68K). A comparison of the mutant and the wild type has been made in order to explain the large effect of this conservative mutation on the affinity.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 35000-29 LMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemical and Structural Investigations of Nucleic Acids and Related Molecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

H. Todd Miles, Ph.D.	Chief, Section on Organic Chemistry	LMB/NIDDK
F. B. Howard, Ph.D.	Research Chemist	LMB/NIDDK
J. Frazier	Research Chemist	LMB/NIDDK
Keliang Liu, Ph.D.	Visiting Fellow	LMB/NIDDK

COOPERATING UNITS (if any)

Girjesh Govil, TIFR, India / Philip Ross LMB/NIDDK  
V. Sasisekharan, Visiting Scientist; Indian Instit. of Science, Bangalore, India  
C-Q. Chen, Biotechnology Institute, Shanghai, China

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Organic Chemistry

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

5

PROFESSIONAL:

5

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have developed a detailed molecular model for the DNA triple helix dT dA dT, in which the helix has three symmetry elements: a pseudodyad relating the Watson-Crick strands, an exact dyad relating the two T strands, and a pseudorotational symmetry relating the Hoogsteen A and T strands. The structure is constrained to a small region of conformation space and has little flexibility. We have obtained crystals of DNA oligonucleotide helices which give fiber type X-ray diffraction patterns. These patterns support our model but indicate that the molecules are ordered only in the axial direction since the usual crystal diffraction spots are not seen.

We have obtained and characterized for the first time two-stranded DNA helices with Hoogsteen base pairing. The structural constraints of the duplex are the same as those encountered in modeling the triple helix, of which the Hoogsteen duplex is a component, and serve to determine the structures of both helices. Nearly identical carbonyl vibration spectra of the Hoogsteen duplex is a component, and serve to determine the structures of both helices. Nearly identical carbonyl vibration spectra of the Hoogsteen duplex and the triple helix (both quite different from that of the Watson-Crick duplex) are consistent with the dyad symmetry relating the two pyrimidine strands.

In reexamining the interaction of poly dT and poly dA we have completed the phase diagram and observed the disproportionation reaction  $2 A T \rightleftharpoons A T_2 + A$ , not previously reported in this system. Both the 2 3 and 3 2 transitions have unusually high salt independence, indicating that the DNA triple helix binds more  $Na^+$  than RNA triplexes, possibly because of closer proximity of the phosphates in the B form DNA helices.

THE JOURNAL OF THE

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36003-9

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nonheritable Antibiotic Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. L. Rosner, Ph.D. Research Biologist LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

We previously reported that growth in salicylate increases the resistance of a number of Gram-negative bacteria to a variety of clinically important antibiotics. We have traced part of this effect in *Escherichia coli* to a cascade of molecular events initiated by induction of transcription of the *marRAB* (multiple antibiotic resistance) operon. One consequence of *mar* induction is increased transcription of *micF* whose mRNA is partly anti-sense to the mRNA of *ompF*. This results in a severe reduction in the translation of *ompF* mRNA into *OmpF*, a major outer membrane porin. The absence of *OmpF* reduces the permeation of the outer membrane by antibiotics. Thus, treatment with salicylate induces the same profile of antibiotic resistance that is found in rare constitutive *mar* mutants.

In addition to the effects on *micF* and *ompF*, induction of *mar* increases the expression of at least 5 other genes including Mn-superoxide dismutase and glucose-6-phosphate dehydrogenase. We have now identified *inaA*, a weak acid inducible gene, as being regulated by *mar*. *mar* constitutive (antibiotic resistant) mutants express *inaA* constitutively. Furthermore, *marA/B* is necessary for the inducibility of *inaA*, but not its basal level expression. Basal level expression of *inaA* requires the presence of other sequences mapped to within 39 kb of *mar*.

Substances that induce *mar* and *inaA* include weak acids (benzoate, acetylsalicylate, acetaminophen); uncouplers (2,4-dinitrophenol), antibiotics (chloramphenicol, tetracycline); and superoxide generators (paraquat, menadione). The nature of the sensor(s) involved in monitoring these seemingly diverse effectors is not known. Compounds that interfere with the sensors could reduce bacterial defense against antibiotics.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36104-12

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thermal Measurements of Biomolecular Systems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Philip D. Ross, Ph.D. Research Chemist LMB/NIDDK  
OTHERS: A. Shrake, Ph.D. Research Chemist DBBP/CPB  
C.P. Mudd, Ph.D. Engineer BEIP/RR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology, DIR/NIDDK

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2

PROFESSIONAL:

2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

On account of renewed interest in triple-stranded nucleic acid complexes we are carrying out a comprehensive thermodynamic study of helix formation among DNA homopolymers containing adenine, thymine and uracil. We have found that dissociation of a third strand of poly(dT) vs. that of poly(dU) from the same poly(dA).poly(dT) double helix over the range of 35°C to 75°C is characterized by a 400 cal/mol greater enthalpy change that is offset by a 1 cal/deg/mol entropy change. The heat capacity change in the reaction was found to be the same for the two different polymer systems. Since the heat capacity change is the hallmark characteristic of the hydrophobic effect, this result indicates that there is little change in solvent exposure of the thymine methyl groups upon dissociation of the third polypyrimidine strand. Since the structures of the two complexes are very likely identical, these experiments establish the magnitude of the energy changes in a well defined comparison between poly(dT) and poly(dU).





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 36105-11

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Influences of Macromolecular Crowding on Biochemical Systems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. B. Zimmerman, Ph.D. Research Chemist LMB/NIDDK

OTHERS: L. D. Murphy Biologist LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory Molecular Biology

SECTION

Section on Physical Chemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.8

PROFESSIONAL:

1.8

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Minors ☐ (b) Human tissues ☐ (c) Neither  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unspaced type. Do not exceed the space provided.)

The high concentration of macromolecules within cells can result in large excluded volume effects. We have studied crowding effects under conditions that approach more closely to such cellular conditions by measuring reactions in the presence of very concentrated extracts from cells of *Escherichia coli*. We find the rate of a test reaction, the cohesion of complementary sequences of lambda DNA, to be accelerated by one or two orders of magnitude under such conditions.

The increased reaction rate is correlated with condensation of the DNA. Condensation requires a DNA-binding protein fraction from the extracts. The amount of DNA-binding proteins causing these results can be decreased greater than 10-fold by concomitantly supplying a background of a purified polymer such as PEG 8000 which provides a moderate level of crowding. We suggest that both cytoplasmic crowding effects and DNA-binding proteins are required to cause the massive condensation of DNA into nucleoids in bacteria, a phenomenon which has remained a poorly understood characteristic of prokaryotes.

A review of macromolecular crowding effects on genome structure and expression is being written for *Biochimica Biophysica Acta*. A more general review on macromolecular crowding in collaboration with Allen Minton was completed. Finally, during the course of this year, we moved our laboratory from Bldg. 2 to Bldg. 5, requiring a significant input of working time.

100

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 36108-06

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Mechanism of Retroviral DNA Integration

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Robert Craigie, Ph.D.	Visiting Scientist	LMB/NIDDK
Frederick Bushman, Ph.D.	Special Volunteer	LMB/NIDDK
Alan Engelman, Ph.D.	IRTA	LMB/NIDDK
Myung Soo Lee	IRTA	LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Genetic Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3.5

PROFESSIONAL:

3.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unnumbered type. Do not exceed the space provided.)

A critical step in the retroviral replication cycle is integration of a DNA copy of the viral genome into a chromosome of an infected cell. The objectives of this project are to understand the detailed molecular mechanism of this step in the retroviral replication cycle. In vitro assay systems developed in this laboratory have enabled us to previously determine many key features of the reaction mechanism. Our work continues to focus on the biochemical activities of HIV-1 integrase protein.

The HIV-encoded integrase protein catalyzes both the 3' processing reaction that cleaves two nucleotides from the 3' ends of the viral DNA prior to integration, and also the subsequent DNA strand transfer step that inserts the viral DNA ends into a target DNA; integrase can also promote an apparent reversal of the DNA strand transfer reaction termed disintegration.

We have constructed an extensive set of point mutations in the HIV integrase gene and purified the mutant proteins. Each protein has been assayed for 3' processing, DNA strand transfer, and "disintegration" activities. The results demonstrate that the central core region of integrase is both necessary and sufficient for the disintegration reaction and therefore contains the active site for polynucleotidyl transfer. However, additional functions supplied by the N-terminal and C-terminal regions of integrase are necessary for the 3' processing and DNA strand transfer activities.

Mutant integrase proteins that have little or no activity when assayed alone complement to restore near wild type levels of activity when assayed as certain pairwise mixtures. This result demonstrates that integrase functions as a multimer in both the 3' processing and DNA strand transfer reactions. Inspection of the pairs of mutant proteins that are able to complement reveals that, within the active multimer, the N-terminal domain of one monomer functions together with the catalytic domain of another monomer.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 36109-6 LMB

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

AIDs Related Proteins: Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: David R. Davies, Chief, Sect. on Molecular Structure LMB/NIDDK

Other: Alison B. Hickman, IRTA LMB/NIDDK  
Frederick Dyda, IRTA LMB/NIDDK  
Enid W. Silverton, Research Chemist LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.2

PROFESSIONAL:

2.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unimproved type. Do not exceed the space provided.)

Several N- and C-terminal truncated forms of the HIV1 integration protein have been cloned, purified and subjected to crystallization attempts. Two forms have demonstrated dramatically improved solubility properties compared to the full-length protein. Solutions have been prepared of a complex of the integration protein and a monoclonal antibody Fab, and crystallization conditions are being explored.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36114-3

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural Studies of Molecular Recognition

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Eduardo A. Padlan, Ph.D.	Visiting Scientist	LMB/NIDDK
Others: Chantal Abergel, Ph.D.	Visiting Fellow	LMB/NIDDK
Jennifer P. Tipper, Ph.D.	Temporary GS-12	LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

3

PROFESSIONAL:

3

OTHER:

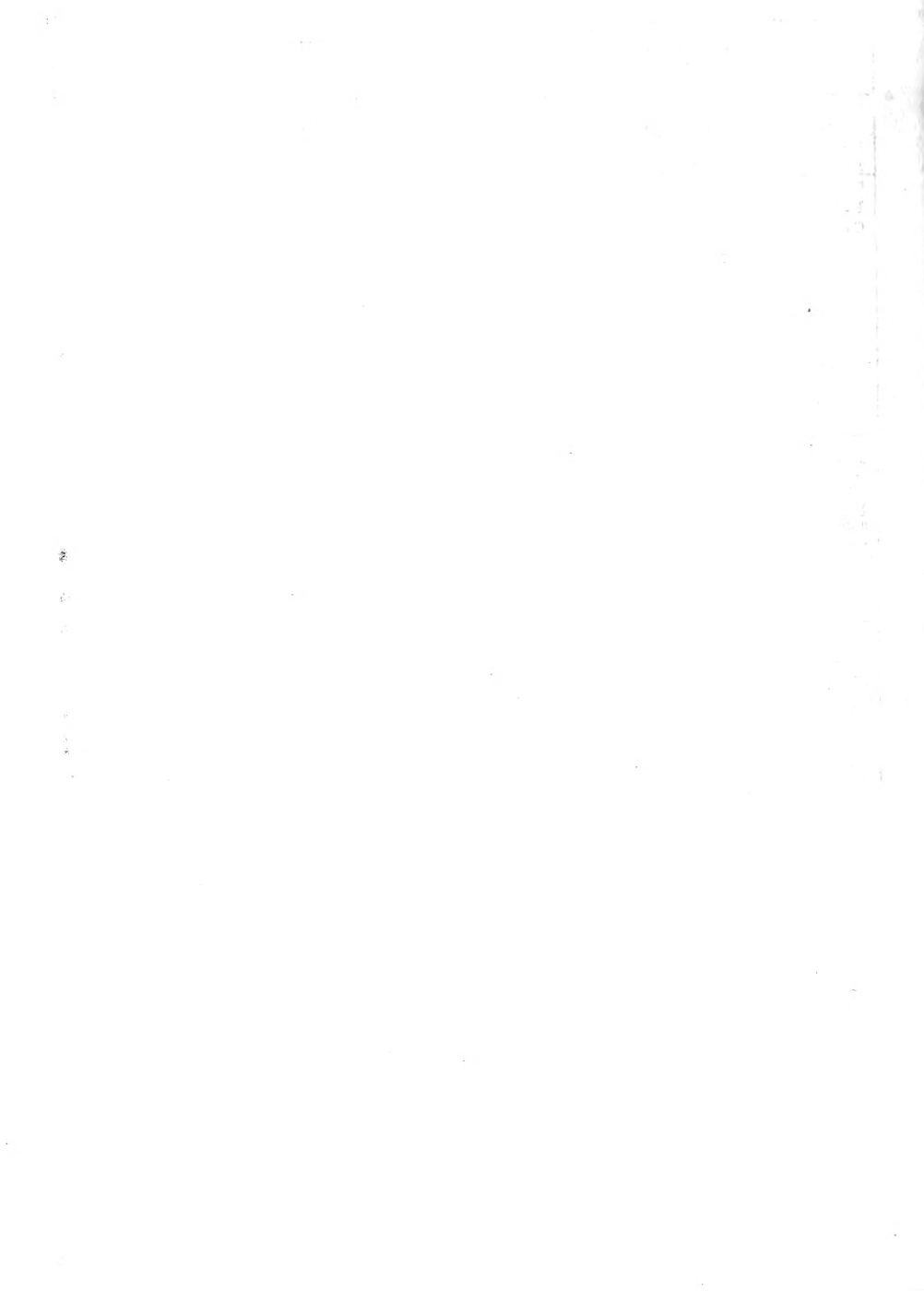
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CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects    ☐ (b) Human tissues    ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

- (1) A molecular replacement solution of the crystal structure of unliganded Fab of HyHEL-10 has been obtained and is being refined.
- (2) The molecular replacement analysis of the crystal structure of the Fab of CC49, a murine monoclonal antibody against solid adenocarcinoma, is being pursued.
- (3) Crystals of a single-chain construct containing the  $V_L$  and  $V_H$  of CC49 fused to human IgG1  $C_H2$  and  $C_H3$  have been obtained.
- (4) Crystallographic studies on various other proteins have been initiated.
- (5) Models of the extracellular portions of the human and murine low-affinity IgE receptors (CD23) have been built.
- (6) The binding of various viral and self peptides to the murine class I MHC antigen, H-2D<sup>d</sup>, has been modelled.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36115-3

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of the Potential Use of Catalytic Antibodies against AIDS

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Eduardo A. Padlan, Ph.D. Visiting Scientist LMB/NIDDK

COOPERATING UNITS (if any)

<u>Ettore Appella, M.D.</u>	<u>Medical Officer</u>	<u>LCB/NCI</u>
<u>Birgit A. Helm, Ph.D.</u>	<u>Lecturer</u>	<u>Univ. of Sheffield</u>
<u>Thomas J. Kindt, Ph.D.</u>	<u>Chief</u>	<u>LIG/NIAID</u>

LAB/BRANCH

Laboratory of Molecular Biology/NIDDK

SECTION

Section on Molecular Structure

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1

PROFESSIONAL:

1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unenriched type. Do not exceed the space provided.)

Two peptidic transition-state analogs, corresponding to segments in the gp120 sequence, have been synthesized and used as immunogens for the production of murine monoclonal antibodies. Studies using supernatants from clones secreting antibody, incubated for varying lengths of time with transition-state analogs and native peptides, suggest that cleavage of the latter does take place.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 36116-02

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Molecular Basis of Antibiotic Resistance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Robert G. Martin, M.D. Medical Officer LMB/NIDDK

OTHERS: Judah L. Rosner, Ph.D. Research Biologist  
LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory Molecular Biology

SECTION

Section on Microbial Genetics

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unruled type. Do not exceed the space provided.)

Mu-lac insertion mutants of E. coli with altered expression in the presence of salicyl alcohol or that fail to exhibit altered antibiotic sensitivity when exposed to salicyl alcohol have been isolated. Five antibiotic-sensitive mutants were selected on the basis that their resistance to nalidixic acid increased only minimally when grown in the presence of salicyl alcohol. Two of these mutants were also hyper-sensitive to nalidixic acid in the presence of sodium salicylate.

The failure of salicyl alcohol or salicylates to induce antibiotic resistance was drug-specific in four of the five mutants. In one, however, salicyl alcohol failed to induce resistance to ampicillin as well as to nalidixic acid. This mutant responded normally to both antibiotics when grown in sodium salicylate. The mutants have been roughly mapped by Hfr crosses and are currently being sequenced.

Over 100 mutants that fail to express  $\beta$ -galactosidase in the presence of salicyl alcohol have been isolated and are being examined. Preliminary data suggests that the genes mutated in this class are principally expressed in mid-log phase and not in early log phase or at saturation density. We are tentatively referring to these genes as mid-life crisis genes. No mutant was found that increased the expression of  $\beta$ -galactosidase in the presence of salicyl alcohol.



ANNUAL REPORT OF THE METABOLIC DISEASES BRANCH  
National Institute of Diabetes and  
Digestive and Kidney Diseases

The general goals of the Branch are to investigate the mechanism of action of hormones controlling ion transport and mineral metabolism and to investigate the immunological and pathological factors mediating kidney disorders. The branch currently includes sections of Mineral Metabolism (Dr. Marx), Endocrine Regulation (Acting Chief, Dr. Spiegel), Renal Cell Biology (Dr. Balow). Integration of these sections is related to common interests in the pathophysiology of metabolic disorders which interface with the kidney. Systems under study include renal and skeletal tissue, transgenic mice, isolated cells (kidney and parathyroid in culture), hormone receptors (beta adrenergic, parathyroid hormone, calcitonin and 1,25-dihydroxy-vitamin D), parathyroid cell growth factors, and T cell and B cell function in disorders of immunoregulation.

#### Primary Hyperparathyroidism and Familial Hypercalcemia

Clinical studies are continuing on primary hyperparathyroidism and its familial variants. Detailed family screening and case findings have produced approximately 85 kindreds for analysis. These studies allowed segregation of the most common familial variants into two distinct disease syndromes - familial multiple endocrine neoplasia type 1 (FMEN I) and familial hypocalciuric hypercalcemia (FHH). FHH was distinguished from FMEN I by 1) virtually a 100% penetrance for hypercalcemia before age 20, 2) milder clinical manifestations - low incidence of recurrent nephrolithiasis or recurrent peptic ulceration, 3) no hypercalciuria, 4) normal basal concentrations of gastrin, and 5) poor response to subtotal parathyroidectomy. Distinction between the two syndromes, both inherited as autosomal dominant traits, is important because in FHH the clinical course is generally milder and subtotal parathyroidectomy is less likely to be beneficial. In several FHH kindreds one or more members have exhibited life-threatening primary hyperparathyroidism in the neonatal period. This may result sometimes from a double dose of the FHH gene. Dispersed parathyroid cells from one severely affected neonate showed a striking decrease in sensitivity of PTH secretion to extracellular calcium. This disorder may reflect mutation in a gene that directs calcium recognition in both the parathyroid and renal tubular cell.

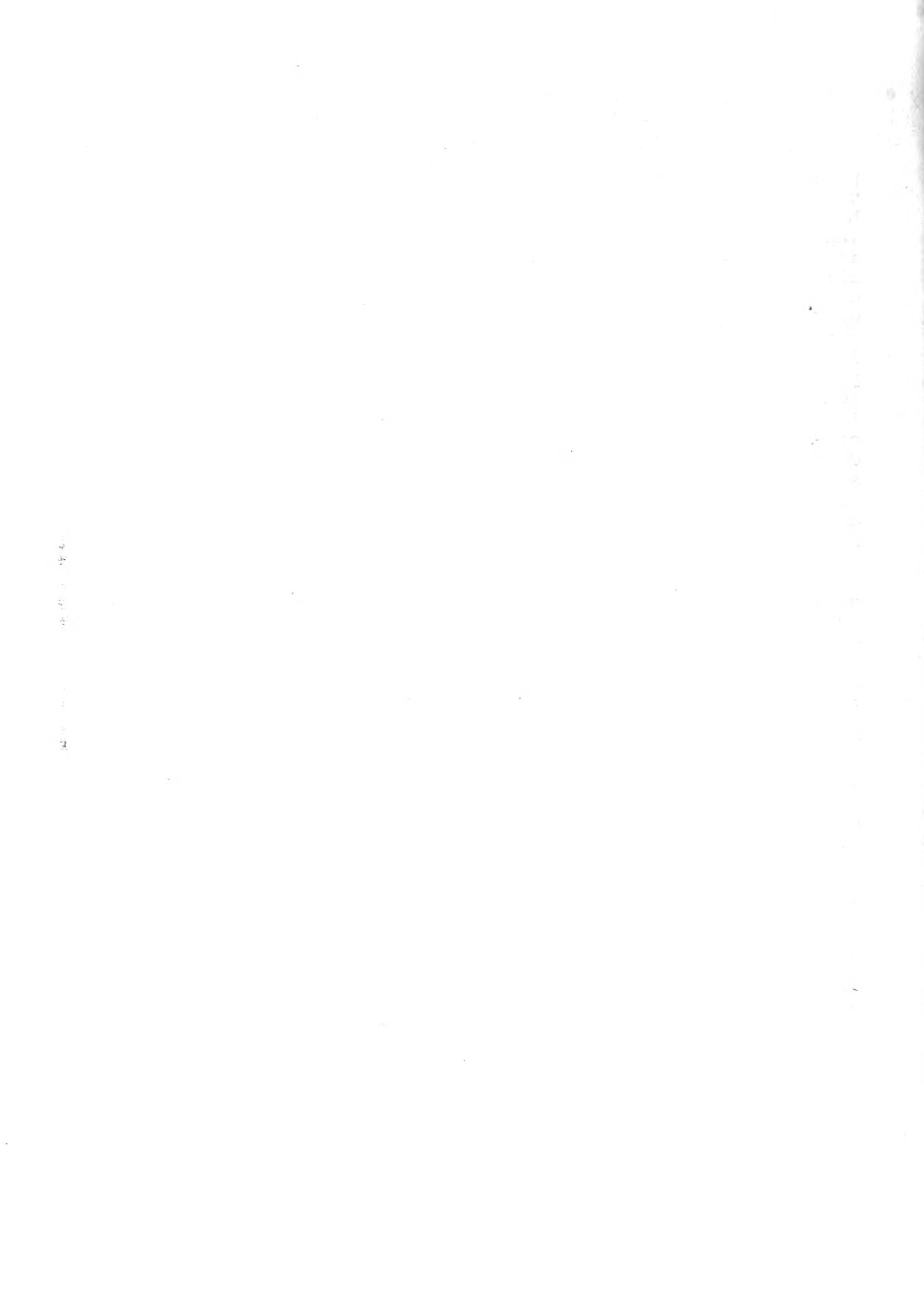
Familial multiple endocrine neoplasia type I (FMENI) is an autosomal dominant disorder characterized by hyperfunction of



parathyroids, pancreatic islets, and anterior pituitary. Affected organs show features suggestive of increased proliferation. Virtually all subjects expressing the gene show primary hyperparathyroidism. Primary hyperparathyroidism is usually first recognizable between ages 20-40, and it shows a high recurrence rate after subtotal parathyroidectomy (approximately 50% after 10 years). We have evaluated multiple indices for use in screening in a very large kindred. We tested 221 members and newly identified 16 as carriers. Albumin-adjusted calcium and PTH were most useful; gastrin and prolactin analyses were not useful for screening but showed promise in followup of known carriers. Analysis in this family has revealed linkage to a locus on the long arm of chromosome 11. The MEN1 gene is a growth suppressor gene like the retinoblastoma gene; MEN1 related tumors are being screened for loss of heterozygosity at this locus. Such loss of heterozygosity has established that most parathyroid tumors in FMEN1 are monoclonal. Tumors with small deletions could speed identification of the MEN1 gene. Similar studies suggest that inactivation of the MEN1 gene also contributes to neoplasia in over 25% of sporadic parathyroid adenomas. [Drs. Friedman, DeMarco, A. Bale, Brandi, Norton, Spiegel, Aurbach, Marx]

With cultured bovine parathyroid cells, we found abnormally high mitogenic activity in plasma from 23 of 27 subjects with FMEN1. Well-characterized growth factors or known parathyroid secretagogues showed far less parathyroid mitogenic activity than these FMEN1 plasmas. The mitogenic factor(s) appears to be a protein of 14,000 mw. We have begun purifying this factor for further characterization. We have obtained evidence that the factor is related to basic fibroblast growth factor. [Drs. Zimering, Brandi, Sakaguchi, Aurbach, Marx].

Studies on noninvasive and invasive modes of localizing parathyroid tumors continue. Parathyroid adenoma localization has been evaluated using the new non-invasive magnetic resonance imaging technique. Initial results were disappointing but the acquisition of a specialized neck collar has led to better resolution in the paratracheal and mediastinal areas. Patients are currently under evaluation with this new technique. A high degree of success has been obtained in localizing tumors through vascular catheterization procedures. Parathyroid arteriography developed and performed by Dr. John Doppman afforded, in approximately 450 of cases tested, the identification of abnormal masses of tissue proven at surgery to be parathyroid. In the most difficult cases, localization of parathyroid tissue can be aided by identifying high concentrations of parathyroid hormone by radioimmunoassay in veins draining the lesion. Fine needle aspiration is another new method that can obviate other invasive localization procedures. We have aspirated with guidance by computerized tomography or ultrasound approximately 20 such lesions that were subsequently confirmed surgically as parathyroid. RIA of the aspirates showed high concentrations of PTH in all but one. Eight mediastinal adenomas have been treated





nonsurgically by percutaneous injection via catheter of occlusive agents into the arterial blood supply with 7 complete and one partial remissions. [Drs. Aurbach, Marx, Spiegel, Weinstein, NIDDK: Dr. Norton, Fraker and Alexander NCI, Drs. Doppman, Miller, and others, Diagnostic Radiology, CC].

### Secretion of Parathyroid Hormone

PTH secretion from parathyroid glands in vivo and cells in vitro is controlled by intracellular calcium and cyclic AMP. Control by calcium is altered in certain pathologic states (glandular adenomas, carcinomas and perhaps hyperplasia). Agents that alter cellular cAMP change PTH secretion in the same direction. Calcium decreases cellular cAMP, but most of its effect to inhibit secretion is independent of changes in cellular cAMP.

Calcium inhibition of parathyroid hormone secretion is controlled through a complex set of mechanisms. We have shown previously that classical voltage-sensitive calcium channels are important in controlling parathyroid hormone secretion and that their action is mediated through a guanine nucleotide regulatory protein. It is also known that the growth of parathyroid cells is controlled by calcium. We have utilized a cloned rat parathyroid cell line (PT-r) to study these phenomena.

Parathyroid hormone synthesis and secretion is well characterized to be stimulated by low calcium. However, there are conflicting data about parathyroid cell growth regulation by calcium. The conflict may have derived from the differences in system and some variables in each system. PT-r cells are clonal and have been proven to show negative regulation of growth by calcium. Previously, we reported that Pt-r cells bear two high affinity receptors for acidic FGF (aFGF), and that at least a subpopulation of the receptors with a higher molecular mass carries heparan sulfate (HS) Glycosaminoglycan chains which give the receptor higher affinity. Now I have found that the parathyroid cell express aFGF, and that aFGF receptors with lower affinity apparently translocate in response to changing extracellular calcium concentrations. Expression of both aFGF mRNA and peptide is suppressed by calcium. Cells have more ligand-accessible receptors on the cell surface at lower calcium concentrations. The apparent translocation of receptors is temperature-dependent but independent of de novo protein synthesis. In concordance with the apparent translocation of aFGF receptors, thymidine incorporation is stimulated by decreasing extracellular calcium concentrations with further stimulation by aFGF. Anti-aFGF antibody inhibits thymidine incorporation by 15% at 0.7 mM  $\text{Ca}^{2+}$  and by more than 30% in the cells exposed to low calcium shortly before adding [ $^3\text{H}$ ]thymidine. An aFGF autocrine system including the apparent translocation of aFGF receptors may explain the mechanism through which parathyroid cell growth is regulated by calcium. [Dr. Sakaguchi]



## Vitamin D Resistance and Related Disorders

The role of  $1,25(\text{OH})_2\text{D}_3$ , the most potent natural metabolite of vitamin D, has been assessed in hypocalcemic states. This very rapidly acting drug has simplified the management of hypocalcemia following parathyroidectomy: during this time skeletal remineralization imposes large but rapidly diminishing requirements for calcium.

We have evaluated patients with extreme resistance to  $1,25(\text{OH})_2\text{D}$ . This can be a transient state as following parathyroidectomy or a permanent state as in familial cases. We have evaluated 20 patients with familial resistance to  $1,25(\text{OH})_2\text{D}$ . Most patients have hypocalcemic rickets, usually with associated total alopecia. The alopecia is associated with the highest grades of resistance to  $1,25(\text{OH})_2\text{D}$ , implicating calcitriol in physiology of the hair follicle. Mineral homeostasis is usually improved by treatments that sustain  $1,25(\text{OH})_2\text{D}$  levels at 10-100 times normal. Intestinal response to  $1,25(\text{OH})_2\text{D}$  can be documented repeatedly with a new stable isotope technique [Drs. Yergey, Viera, Marx].

Specific intracellular defects have been evaluated using cultured skin fibroblasts from these patients. With skin fibroblasts cultured from normals, properties of the  $1,25(\text{OH})_2\text{D}$ -receptor can be identified by binding in soluble extracts, by nuclear uptake of hormone with intact cells, or by elution of occupied receptor from DNA-cellulose. Fibroblasts from patients with familial resistance to  $1,25(\text{OH})_2$  have shown a spectrum of defects including nonfunctional receptors, diminished numbers of receptors, and receptors with decreased hormone binding affinity. Among cases with normal hormone binding sites on the receptors some show receptors with deficient binding to nucleus while others show normal binding to nucleus but abnormal interaction with nonspecific DNA (as DNA-cellulose). Cellular action of  $1,25(\text{OH})_2\text{D}_3$  can be analyzed by measuring its induction of the  $25(\text{OH})\text{D}$  24-hydroxylase enzyme system. Cultured skin fibroblasts from all patients with hereditary resistance to  $1,25(\text{OH})_2\text{D}$  exhibit defects in this induction. Immunocytology reveals multiple rapid steps of reorganization of vitamin D receptors after calcitriol addition. Specific disruptions in these steps can be imaged in mutant cells from patients. Four different homozygous, point mutations in the gene for the Vitamin D receptor have so far been identified as the cause of this disorder in six kindreds. [Drs. Marx, Barsony, MDB, NIDDK; Dr. Liberman, Israel; Drs. Pike (Baylor) and DeLuca (Madison)].

Fibroblast lines from patients with hereditary extreme resistance to  $1,25(\text{OH})_2\text{D}_3$  are being used to probe for normal functions of the  $1,25(\text{OH})_2\text{D}_3$  receptor. We have shown that  $1,25(\text{OH})_2\text{D}_3$  can elevate intracellular cyclic GMP very rapidly (within 1-3 minutes). This response showed affinity and analog specificity characteristic of the  $1,25(\text{OH})_2\text{D}_3$  receptor and was



absent in all "mutant" fibroblast lines although they retained a rapid cGMP response to nitroprusside and to androgens. Thus a  $1,25(\text{OH})_2\text{D}_3$  receptor mediates this rapid response. Immunocytochemistry revealed that, after  $1,25(\text{OH})_2\text{D}_3$  addition, cGMP accumulates rapidly about the reorganizing vitamin D receptors [Drs. Barsony, Marx].



RENAL CELL BIOLOGY SECTION  
Studies of the pathogenesis of glomerulosclerosis

The renal cell biology section is interested in the cellular and molecular mechanisms leading to glomerular scarring, the emphasis being on non-immune diseases. The kidney disease of diabetes mellitus is the major disease studied. Our hypothesis is that glomerulosclerosis results from abnormalities in the rate of proliferation and matrix turnover of resident glomerular cells. This is being investigated in vitro, using lines of glomerular cells, and in vivo using transgenic mice. We are also currently studying specimens from human nephrectomies. We have developed a new technique to quantitatively study the synthesis and degradation of glomerular matrix components in vivo. The method consists of microdissection of single glomeruli, reverse transcription in situ followed by competitive polymerase-chain reaction (PCR). We are examining the relative amounts of mRNAs coding for the various basement membrane components and for enzymes that degrade collagens in glomeruli of mice transgenic for growth hormone, in diabetic mice, and in human glomeruli obtained from nephrectomy specimens. In addition we have been examining whether injection of advanced glycosylation end products increases glomerular basement membrane synthesis since this may provide a direct estimation of the adverse consequences of hyperglycemia on the kidney. Current investigators are: LJ Striker, GE Striker, EP Peten, Ci-Jiang He, CW Yang, C Linder.

I. Glomerulosclerosis

A. In Vivo Studies.

1. Mice transgenic for GH: Mice transgenic for GH develop severe glomerulosclerosis with a disproportionate increase in the size of the glomeruli. Since the morphological appearance of the lesions mimics diabetic glomerulosclerosis we have used this model as a tool to examine the local events leading to glomerulosclerosis. We found an increase in mRNAs coding for collagen types I and type IV in glomeruli as well as metalloproteinase 72KD, using a method consisting of microdissecting mouse glomeruli followed by a quantitative analysis of polymerase chain reaction products. In mice transgenic for mutated GH species it was found that body size and glomerulosclerosis are regulated in an independent manner. (L. Striker, E. Peten, CW. Yang, G. Striker)

2. Nonobese diabetic (NOD) mice: NOD mice develop an autoimmune diabetes mellitus. We compared glomerular size, morphology, composition of the sclerotic extracellular matrix, and urine protein excretion rate in mice before and after the onset of diabetes mellitus. An increase in glomerular size, mesangial sclerosis, and proteinuria rapidly followed the onset of hyperglycemia. We are synchronizing the onset of clinical diabetes mellitus with streptozotocin injections and assessing matrix synthesis in isolated glomeruli. These mice could provide a good model of nephropathy in a genetically determined model of IDDM. (CW. Yang, L. Striker, G. Striker)





### 3. Expression of genes coding for collagens and degradative enzymes in Human Glomeruli

The expression of several genes involved in glomerulosclerosis has been investigated in human microdissected reverse-transcribed glomeruli, using competitive PCR. In patients with and without glomerulosclerosis, we have begun comparisons of mRNAs coding for 1) the  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  chains of type IV collagen; and 2) The tissue inhibitors of metalloproteinases, TIMP I and TIMP II. These species are all present in higher amounts in sclerotic, compared to normal glomeruli. This method provides the first quantitative means to study the amounts of types of molecules responsible for the molecular events leading to glomerulosclerosis in man. (L. Striker, G. Striker, E. Peten, CW. Yang,).

4. Glomerular size is genetically determined. We have undertaken a retrospective study of the glomerular lesions in diabetic Pima Indians. We developed morphometric methods to measure glomerular size to determine whether glomerulosclerosis is associated with hypertrophy. We found that Pima Indians have large glomeruli, and that glomerular size in Pimas with diabetes mellitus did not differ from non-diabetics. The size of glomeruli in African Americans and Caucasians was also determined, utilizing forensic autopsies. Glomerular size paralleled the incidence of endstage kidney disease in these populations, i.e., Pima>African Americans>Caucasians. (L. Striker, P. Bennett, G. Striker, C. Pesce, K. Schmidt)

### B. In Vitro Studies.

1. Murine glomerular cells: We have developed lines of mouse epithelial, mesangial, and endothelial cells from normal mice and from several strains of transgenic mice and have been investigating their response to growth peptides and advanced glycosylation endproducts. Mesangial cells synthesize collagen types IV and I as well as metalloproteinases I and their inhibitor (TIMP I). When these cells are exposed to advanced glycosylation endproducts, there is a rapid increase in collagen synthesis, providing evidence that these products may be one cause of glomerulosclerosis in diabetes mellitus. To investigate the phenotype of mesangial cells in culture we have developed a new culture system using methylcellulose. The phenotype of mesangial cells plated on methylcellulose is closer to the in vivo situation than that of cells plated on plastic or fibronectin. (Ci-Jiang He, M. Carome, E. Peten, L. Striker, G. Striker)

2. Analysis of lines of mesangial cells from mice that develop glomerulosclerosis: We developed mesangial cell lines from NOD mice and from mice transgenic for bovine growth hormone. The NOD cells exhibited less surface receptors for IGF-I after the occurrence of diabetes but released more peptide in the supernatant. The phenotypic changes induced by diabetes may participate in the development of glomerular abnormalities. (L. Striker, E. Peten, Ci-Jiang He, S. Elliot, G. Striker)



## KIDNEY DISEASE SECTION

The Kidney Disease Section conducts parallel clinical and laboratory research centered on human glomerular diseases, experimental models of immune-mediated renal disorders, mechanisms of immunosuppression, and normal biology of various types of kidney cells. Patient and animal tissues are used to study pathogenetic mechanisms, primarily involving immune functions, cytokines and growth factors. The effects of various immunosuppressive drugs at the level of gene regulation and transcription and novel immunosuppressive drug therapies which might have salutary effects on the course of lupus nephritis and membranous nephropathy are under study.

### Membranous nephropathy.

Membranous nephropathy causes an insidious loss of renal function in patients with lupus and in those patients with idiopathic forms of this disease. Current protocols involve examination of the pathophysiology of the glomerular lesions in membranous nephropathy, as well as evaluation of the comparative efficacy of prednisone, cyclophosphamide and cyclosporin A in patients idiopathic and lupus-related forms of this renal disease. (Balow, Austin, Boumpas, MacKay).

### Studies of new immunosuppressive agents for glomerular diseases.

Previous studies have shown that intermittent pulse cyclophosphamide therapy is superior to conventional prednisone in management of lupus nephritis. Our group has further shown that pulse cyclophosphamide is significantly more effective than pulse methylprednisolone in reducing lupus activity and the risk of progressive renal failure.

Although pulse cyclophosphamide is considered by most to be the current standard of therapy for severe lupus nephritis, its effectiveness is not optimal and gonadal toxicity is a limiting complication. In collaboration with NIAMS, we are initiating studies of newer nucleoside analogues in patients with glomerulonephritis, utilizing therapeutic strategies which produced substantial immunosuppression without major toxicity in protocols for treatment of B cell lymphomas.

Chlorodeoxyadenosine and fludarabine are agents with distinctive selectivity for lymphocytes; this is due to their particularly high levels of deoxycytidine kinases which are necessary for activation of these drugs. We are conducting pilot studies of chlorodeoxyadenosine in lupus nephritis and fludarabine in refractory membranous nephropathy. We plan to initiate a head-to-head comparison of pulse cyclophosphamide and these nucleoside analogues. We are also planning additional protocols for their use in focal glomerulosclerosis which have proven refractory to standard therapy. (Balow, Austin, Boumpas, MacKay).



### Immunopathogenesis of glomerulonephritis.

Murine models are being utilized to investigate the different components of lupus nephritis. The modulating effects of cyclophosphamide on immune responses in normal mice and on the renal lesions of nephritic mice are being investigated. Studies of differences among the murine strains have provided new approaches to study of the diverse manifestations and response to treatment of human lupus nephritis. (Austin, Patel, Balow).

### Regulation of lymphocyte gene expression.

Dysregulated cell mediated immune responses are present in subjects with most forms of glomerulonephritis. Studies of the mechanisms of control of B and T cell activation, including regulation of immunoglobulin and cytokine genes by nuclear factors are being pursued. Special emphasis is placed on studies of the effects of various immunoregulatory agents, such as corticosteroids, cyclosporine, and cyclophosphamide. (Boumpas, Paliogianni, Ahuja, Balow).

### Transforming growth factor and glomerular reactions.

The mechanisms responsible for normal growth and for pathogenic cellular reactions within the glomerulus are poorly understood. The signal transducing agent, transforming growth factor-beta (TGF- $\beta$ ), has a complex interaction with glomerular cells. Studies are underway to characterize the nature of the receptors for this growth factor on glomerular cells. Several parameters, such as proliferation, fibronectin secretion and proteoglycan synthesis, will be used to evaluate the responses of these cells to binding of TGF- $\beta$ . Studies of factors which regulate TGF- $\beta$  receptors are underway. Information learned from these experiments should facilitate studies of the role of the TGF- $\beta$  ligand and its receptors in experimental models of glomerulonephritis. (MacKay).



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43002-27 MD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Structure, Secretion and Mechanism of Action of Parathyroid Hormone

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A.M. Spiegel, M.D.	Chief	MDB, NIDDK
OTHERS:	S. Doi, M.D.	Visiting Associate	MDB, NIDDK
	K. Sakaguchi, M.D., PhD.	Visiting Associate	MDB, NIDDK
	D. Coleman, PhD.	IRTA	MDB, NIDDK
	Y. Takagi, M.D.	Visiting Fellow	MDB, NIDDK

COOPERATING UNITS (if any)

Endocrine Unit, Massachusetts General Hospital  
National Institute of Dental Research, BRB

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Endocrine Regulations Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

4.5

PROFESSIONAL:

3.5

OTHER:

1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It is the purpose of this project to study the secretion, function, and mechanism of action of parathyroid hormone, its relationship to human disease, and to develop clinically useful tests for circulating parathyroid hormone. From these studies it is expected that one can understand the pathophysiology of certain metabolic diseases of bone and endocrine disturbances. The entire structures of bovine, porcine, rat and human parathyroid hormone have been determined. Synthetic polypeptides representing bovine rat and human parathyroid hormone have been synthesized. These molecules show all the biological properties of the native hormonal polypeptides. Highly sensitive radioimmunoassays for the hormone have been developed and are being modified further for improved clinical diagnostic parameters. Studies show that the mechanism of action of the hormone is mediated through direct hormonal activation of adenylate cyclase in bone and kidney. Isolated parathyroid cells and culture systems have been developed that allow studies on secretory control of parathyroid hormone, and provide test systems to elucidate the pathophysiology of certain hypoparathyroid and hyperparathyroid states.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43003-27 MD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Studies on the Mode of Action of Thyrocalcitonin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S.J. Marx, M.D. Chief, Min. Metab. Sect. MDB,NIDDK

OTHERS: J. Barsony, M.D. Visiting Scientist MDB,NIDDK

COOPERATING UNITS (if any)

NONE

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Mineral Metabolism Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

The purpose is to study the interaction of calcitonin with its specific receptor target organs. The current investigations should provide further insight into the structure-function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful for characterizing hormone receptors in kidney, bone and other tissues. Calcitonin increases cAMP in MCF 7 breast cancer cells. At 300-fold lower concentration calcitonin decreases cAMP in these cells. The decrease in cAMP is prevented by preexposure of cells to agents that interfere with inhibitory guanyl regulatory proteins. Intracellular compartmentalization of cAMP accumulation after calcitonin has been imaged after microwave fixation of cells. The cAMP accumulates initially along the plasma membrane but within 1 to 3 minutes accumulates much closer to the nucleus.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43006-17 MD

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Study of Hyperparathyroidism: Etiology, Diagnosis and Treatment

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S.J. Marx, M.D. Chief, Min. Metab. Sec. MDB, NIDDK

OTHERS: K. Sakaguchi, M.D., PhD Visiting Associate MDB, NIDDK  
A. Spiegel, M.D. Chief MPB, NIDDK  
W. McKoy Chemist MDB, NIDDK

COOPERATING UNITS (If any)

Radiology Department, CC: (J. Doppman, T. Shawker)  
Surgery Branch, NCI; (DL Graker, R. Alexander)  
Department of Endocrinology, Univ. of Florence, Italy (ML Brandi)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Endocrine Regulation Section

INSTITUTE AND LOCATION

NIDDK/NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

0.8

OTHER:

0.2

CHECK APPROPRIATE BOXES

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

The project goal is the evaluation and treatment of hyperparathyroidism. Patients with persistent or recurrent hyperparathyroidism are referred for evaluation and treatment. Hereditary hyperparathyroidism in particular is under investigation in the hopes of delineating hereditary molecular abnormalities in glandular regulation, as exemplified in the multiple endocrine neoplasia syndromes. Evaluation ranges from epidemiologic studies of families to in-house clinical studies of patients and to in vitro analyses of excised tissue. Techniques currently being employed and improved include radioimmunoassay of parathyroid hormone, ultrasonography, (reoperative and intra operative) radiothallium scanning, magnetic resonance imaging, CAT scanning, selective arteriography and selective venous sampling for parathyroid hormone, parathyroid gland cryopreservation and autotransplantation, and transcatheter parathyroid gland infarction. In vitro evaluation of parathyroid and other endocrine tissue involves tissue culture, chemistry and determination of linkage with DNA or RNA probes.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43008-11 MD

PERIOD COVERED  
October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)  
Vitamin D Resistance and Related Disorders

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S.J. Marx, M.D. Chief, MIn. Metab. Sec. MDB, NIDDK  
OTHERS: J. Barsony, M.D. Visiting Scientist MDB, NIDDK  
W. McKoy Chemist MDB, NIDDK

COOPERATING UNITS (if any)

Metabolism Unit, Beilinson Hospital, Betah Tiva, Israel (Uri Liberman)  
Ligand Pharmaceuticals (J.W. Pike)  
Biochemistry Dept., Univ. Of Wisconsin, Madison (HF DeLuca) Hormone Action and

LAB/BRANCH Oneogenesis Section NCI (G. Hager)

Metabolic Diseases Branch

SECTION

Mineral Metabolism Section

INSTITUTE AND LOCATION

NIDDK: NIH: Bethesda, MD 20892

TOTAL STAFF YEARS:

2.5

PROFESSIONAL:

1.7

OTHER:

0.8

CHECK APPROPRIATE BOXES!

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unmodified type. Do not exceed the space provided.)

With recognition that vitamin D is the precursor for 1,25-dihydroxyvitamin D, it has become possible to characterize defects in the activation (1-hydroxylation) of vitamin D and defects in the target action of activated (1,25-dihydroxy) vitamin D. We have demonstrated a broad spectrum of manifestations of hereditary resistance to 1,25(OH)<sub>2</sub>D ranging from infantile rickets with alopecia and no intestinal response to calciferols to adult onset osteomalacia with satisfactory intestinal response to high doses of calciferols and with no epidermal abnormalities. This syndrome usually results from a mutation in the gene for the vitamin D receptor. Skin fibroblasts from all subjects with hereditary resistance to 1,25(OH)<sub>2</sub>D display abnormalities in this effector system, and defects in many discrete steps of this pathway have been identified with these cells. Cells with mutations in the 1,25(OH)<sub>2</sub>D effector pathway can be used to explore mechanisms of calciferol action. They have been used to establish that the 1,25(OH)<sub>2</sub>D receptor mediates an extremely rapid (1-3 minutes) rise of cyclic GMP in response to 1,25(OH)<sub>2</sub>D<sub>3</sub> and that certain receptor mutations compromise many receptor functions but allow another function to be retained normally. This establishes that 1,25(OH)<sub>2</sub>D receptors couple to different responses by distinct mechanisms.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43009-07

PERIOD COVERED

October 1, 1992 to September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Mineral Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S.J. Marx, M.D. Chief, Min Metab Sec MDB, NIDDK

OTHERS: W. McKoy Chemist MDB, NIDDK  
E. Friedman, M.D. Visiting Fellow MPB, NIDDK  
A. Spiegel, M.D. Chief MPB, NIDDK  
M. Skarulis, M.D. Sr. Staff Fellow MDB, NIDDK  
D. Fraker, M.D. Surg., NCI  
R. Alexander, M.D. Surg., NCI

COOPERATING UNITS (If any) EEB, SB, NCI, MPB

Belvedere Medical Center- Carlisle, PA (J. Green), Dept of Physiol, Univ. of  
Manitoba, Canada (J.G. Friesen), Genetics Department - Yale University (A. Bale)  
Dept. of Endo. - Univ. of Florence Italy (ML Brandi) - Brigham Hosp. & Harvard Med.

LABORATORY (Name, title, laboratory, and institute affiliation)

Metabolic Diseases Branch

SECTION

Mineral Metabolism Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

0.8

OTHER:

0.2

CHECK APPROPRIATE BOXES:

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.)

Disorders of mineral metabolism have been evaluated with methods extending from epidemiology to cellular and molecular biology. Two forms of familial hyperparathyroidism have been characterized in detail. Familial hypocalciuric hypercalcemia is an autosomal dominant trait associated with abnormal interactions with calcium in parathyroid and kidney. Familial multiple endocrine neoplasia type 1 (FMEN1) is an autosomal dominant trait causing hyperfunction of parathyroids, pancreatic islet and anterior pituitary. It is associated with gradual but abnormal proliferation of the tissues affected. Genetic linkage studies in a large kindred have localized the FMEN1 gene to the long arm of chromosome 11. Plasma from affected persons shows high mitogenic activity upon cultured bovine parathyroid cells. This mitogenic activity in plasma may contribute to primary hyperparathyroidism in FMEN1. Analysis of blood and parathyroid tumor DNA has revealed that FMEN1 parathyroids often show clonal loss of alleles in the region of the FMEN1 gene on chromosome 11. Thus the FMEN1 gene probably functions as a tumor suppressor gene, analogous to the retinoblastoma gene. Analysis of sporadic parathyroid adenomas revealed that 25% showed allelic loss in a similar region. Thus the clonal inactivation of the FMEN1 gene





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
**NOTICE OF INTRAMURAL RESEARCH PROJECT**

PROJECT NUMBER

Z01 DK 43204-13 MD

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Immunosuppressive Drug Therapy in Lupus Glomerulonephritis**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: J.E. Balow Senior Investigator MDB, NIDDK  
Others: H.A. Austin III Medical Officer MDB, NIDDK  
D.T. Boumpas Visiting Scientist MDB, NIDDK

COOPERATING UNITS (if any)

NIAMS (D. Kastner, J. Klippel, P. Plotz, R. Wilder)  
CC (E. Vaughan, C. Yarboro)

LAB/BRANCH

**Metabolic Diseases Branch**

SECTION

**Kidney Disease Section**

INSTITUTE AND LOCATION

**NIDDK, NIH, Bethesda, MD 20982**

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Pulse cyclophosphamide is more effective than prednisone alone in preventing renal failure in lupus nephritis. This study sought to define whether pulse methylprednisolone could equal pulse cyclophosphamide in preserving renal function, and whether there was a difference between long and short courses of pulse cyclophosphamide in preventing exacerbations of lupus.

Patients were treated with prednisone and randomized to receive concomitantly (a) pulse methylprednisolone monthly for 6 months, or (b) pulse cyclophosphamide monthly for 6 months, or (c) pulse cyclophosphamide monthly for 6 months followed by a maintenance regimen every 3 months for an additional two years.

Patients treated with pulse methylprednisolone had a higher probability of developing renal insufficiency than patients treated with the long course of pulse cyclophosphamide. In addition, patients treated with only a short course of pulse cyclophosphamide had a higher probability of major exacerbations of lupus than those treated with the extended course of pulse cyclophosphamide.

Studies of ovarian toxicity in patients treated with pulse cyclophosphamide showed that risk is affected by both age at treatment and total doses of cyclophosphamide.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43205-16 MD

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Renal Biopsy Pathology in Systemic Lupus Erythematosus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: J.E. Balow Senior Investigator MDB, NIDDK  
Others: H.A. Austin III Medical Officer MDB, NIDDK

COOPERATING UNITS (if any)

Armed Forces Institute of Pathology, Washington, DC (T. Antonovych,  
S. Sabnis); CC (E. Vaughan)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20982

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Diverse pathogenetic factors are operant in systemic lupus erythematosus and lead to different forms of lupus nephritis. Detailed analysis of renal biopsy pathology is being conducted on specimens from patients with systemic lupus erythematosus.

Biopsies are classified by standard major category of lupus nephritis, as well as scored on a semi-quantitative scale for specific histologic changes which indicates the extent and severity of active inflammatory lesions and chronic atrophic, fibrosing and sclerosing features. The patterns of immune complex deposition and lymphoid cell interaction with different segments of the nephron are being investigated by immunohistologic techniques and electron microscopy.

These approaches have facilitated the analysis of the effects of various types of immunosuppressive agents used to halt the progression of lupus nephritis and they will enhance our understanding of the pathogenesis of lupus nephritis.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43211-09

PERIOD COVERED

10/1/92 thru 9/30/93

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Morphometry of the Glomerulus in Pimas and other Minority Populations

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: GE Striker, Senior Investigator

Others: LJ Striker, Chief RCBS

COOPERATING UNITS (if any)

P Bennett, Chief ECR, Phoenix, AZ

A Fogo, Dept. Path, Vanderbilt U, Nashville, TN

C M Pesce University of Genova Italy

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bldg. 10, Rm. 3N-110

INSTITUTE AND LOCATION

NIIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.10

PROFESSIONAL:

0.10

OTHER:

CHECK APPROPRIATE BOX(ES)

- (a) Human ☒ (b) Human ☐ (c) Neither ☐  
(a1) Minors ☐  
(a2) Interviews ☐

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The mean of the area and a size-class distribution of the glomeruli were calculated with a computer-assisted planimeter in a series of autopsy specimens of diabetic and non diabetic Pima Indians. These morphometric variable were also related to the severity of histologic glomerular lesions, graded on a four class scale. Glomerular size did not differ in the two groups, nor were there differences in the size distribution discernible among the classes of histologic lesions. Therefore in the Pima Indians the occurrence of IDDM did not result in an increase in the glomerular volume. This morphometric analysis was compared with that obtained from a series of forensic autopsies from Caucasians matched for age and sex. The mean glomerular volume was greater in the Pima Indians than in the Caucasians. Furthermore the volume fraction of the cortex occupied by the glomeruli was greater in the Pima Indians, suggesting that their increased glomerular volume was not due to a reduced number of glomeruli. The large size of the glomeruli in the Pima Indians was independent of obesity or heart weight and could constitute a genetic trait of this population. In this population the changes in glomerular mass and filtration surface may underlie a familial predisposition to developing renal disease. Finally we undertook a similar study in a series of forensic autopsies in African Americans since their incidence of end stage renal disease is much higher than in the caucasian population. The mean size of the glomeruli was significantly greater in the African American population. We postulate that the increased glomerular size may constitute a marker for a sclerosis-prone population



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

43214-09 MD

PERIOD COVERED

10/01/92 thru 9/30/93

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Glomerular Cells Derived from Normal and Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: GE Striker, Senior Investigator

Others: LJ Striker, Chief RCBS

Ci-Jiang HE, Visiting Associate

E P Peten, Visiting Associate

S Elliot, GS 12

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Building 10, Room 3N-110

INSTITUTE AND LOCATION

NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.12

PROFESSIONAL:

1.12

OTHER:

CHECK APPROPRIATE BOX(ES)

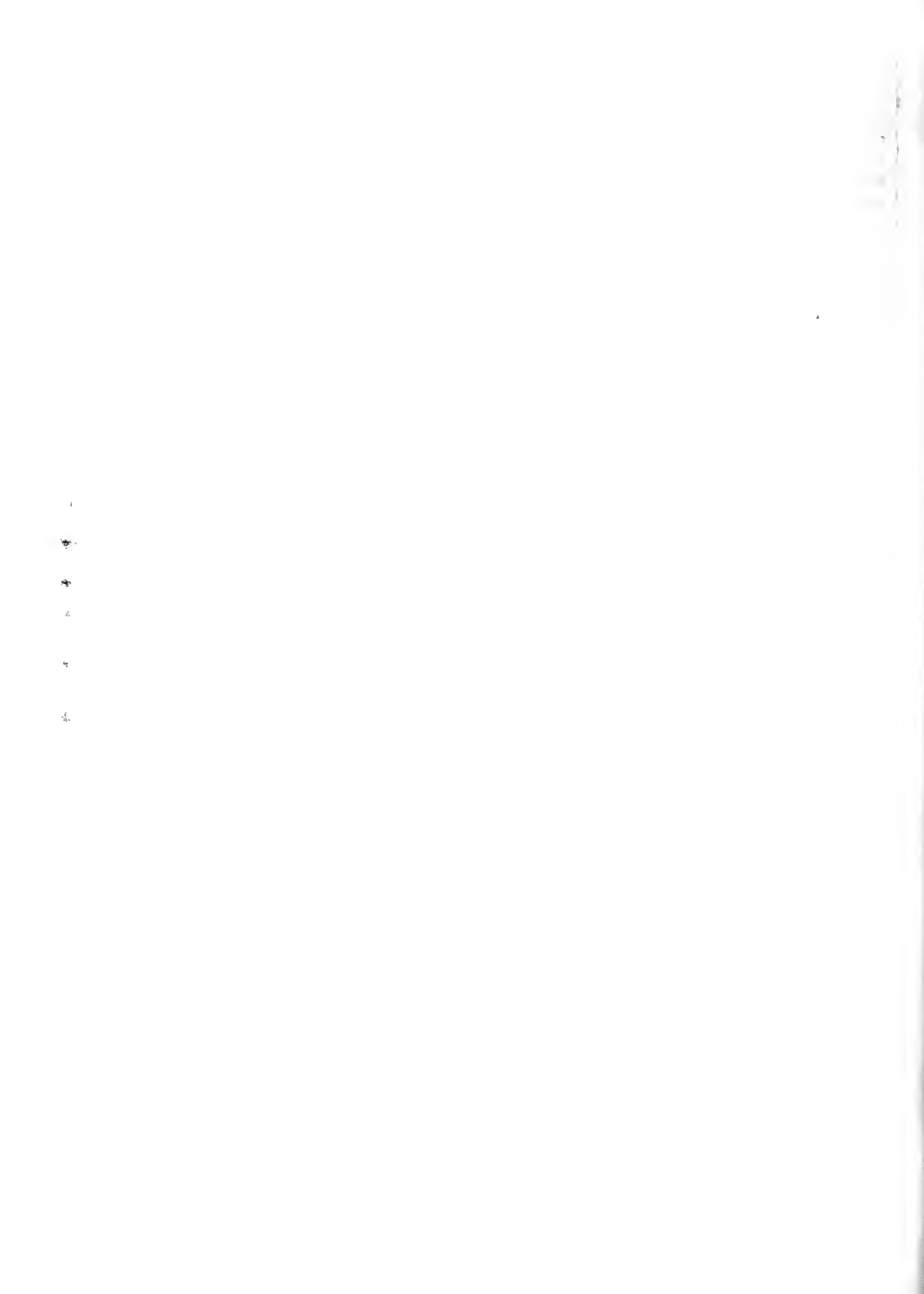
(a) Human (b) Human X (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Glomerulosclerosis, characterized by increased glomerular cell turnover and increased turnover of extracellular matrix turnover, is the leading cause of renal failure in the US. Current studies in humans or animal models of glomerulosclerosis (GS) have yielded little information about the cellular and molecular abnormalities that are critical in the initiation and progression of this disease. This is due, in part, to the difficulty in isolating and characterizing glomeruli in vivo, and the fact that the glomerulus contains three indigenous cell types and a population of bone marrow-derived macrophages. This is particularly a problem with mice, a species we have chosen to study because of the availability of considerable genetic information about extracellular matrix and growth regulatory factors. We have approached this problem by isolating the individual cell types and characterizing them in vitro. The sources of the cells types examined include normal mice, non-obese diabetic mice, and mice transgenic for bovine growth hormone. We first examined the question of the phenotypic modulation of glomerular mesangial cells in vitro, addressing the question of whether this occurred, what was the time frame of the occurrence, and whether there was a difference between mesangial cells from different isolates or different sources. We found that there was a marked difference between cells at different passages, as well as between different isolates, and different mouse strains.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43222-08 MD

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis of Murine Lupus Nephritis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.:	H. A. Austin	Medical Officer	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	D. T. Boumpas	Visiting Scientist	MDB, NIDDK
	A. D. Patel	Biologist	MDB, NIDDK

COOPERATING UNITS (if any)

Armed Forces Institute of Pathology; Washington, DC (T. Antonovych and S. Sabnis)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.6

PROFESSIONAL:

0.3

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects   ☐ (b) Human tissues   ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Investigations of the pathogenesis and treatment of lupus nephritis are facilitated by the availability of inbred strains of mice that develop disease similar to human systemic lupus erythematosus. The natural evolution of the diverse histologic features of murine lupus nephritis has been studied to delineate the types of glomerular and tubulointerstitial lesions.

Innovative treatment strategies will be studied to refine our approach to this disease. The impact of biologic response modifiers on immunologic features will be investigated as well. Clinical, histologic and immunologic outcome parameters will be evaluated including detailed studies of renal morphology, and the characteristics of spleen lymphocytes employing flow cytometry, measures of immunoglobulin gene expression, and in vitro assays of alterations in humoral and cell mediated immune regulation.

An investigation of the effects of monthly doses of intraperitoneal cyclophosphamide in NZB/W female mice has been undertaken. Multiple doses of "pulse" cyclophosphamide lead to prolonged effects on the numbers (total and phenotypes) and the functions (spontaneous immunoglobulin secreting cell responses) of lymphocytes (spleen and peripheral blood). Additional studies regarding the mechanisms underlying these effects are in progress.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43224-07 MD

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Membranous Lupus Nephropathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	H. A. Austin	Medical Officer	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	K. MacKay	Expert	MDB, NIDDK

COOPERATING UNITS (if any)

CC (E. Vaughan); NIAMS (J. Klippel); Stanford University; Stanford, CA (B. Myers).  
Armed Forces Institute of Pathology, Washington, DC (T. Antonovych and S. Sabnis).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.4

PROFESSIONAL:

0.4

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects   ☐ (b) Human tissues   ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It is currently unknown whether therapeutic intervention will alter the course of membranous lupus nephropathy. In the present study, the efficacy and toxicity of three immunosuppressive drug regimens administered over a 12 month period will be evaluated in patients with membranous lupus nephropathy. Detailed tests of renal function (including radiolabelled compounds for glomerular filtration and renal plasma flow rates), glomerular permselectivity (using fractional clearance of graded dextrans) and kidney biopsy morphology will be performed at the beginning and end of treatment. Patients with systemic lupus erythematosus, nephrotic range proteinuria and biopsy documented membranous nephropathy will be randomized to receive: a) alternate day prednisone alone (control group), b) alternate day prednisone plus intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses, or c) alternate day prednisone plus oral cyclosporin A up to 200 mg per square meter body surface area daily. Lupus disease activity, renal function tests and drug toxicities will be monitored closely. Analysis will include comparison of the numbers of favorable outcomes of glomerular filtration rate, renal plasma flow, permselectivity, glomerular pathology and drug-related toxicities appearing in each treatment group.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43225-06

PERIOD COVERED

10/1/92 thru 9/30/93

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glomerular Lesions in Mice Transgenic for Growth Hormone

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: LJ Striker, Chief RCBS

Others: GE Striker, Senior Investigator

EP Peten, Visiting Associate

CW Yang, Visiting Associate

COOPERATING UNITS (if any)

John Kopchick, University of Ohio

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bldg. 10, Rm. 3N-110

INSTITUTE AND LOCATION

NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.98

PROFESSIONAL:

0.98

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have shown that mice transgenic for growth hormone develop a rapidly progressive glomerulosclerosis. The lesions resemble those occurring in Diabetes mellitus. Mice transgenic for GH molecules containing mutations have also been examined in order to dissociate the domains of GH that may specifically code for genes responsible for glomerulosclerosis from those coding for body mass increase. We found an upregulation of mRNA for extracellular matrix components in the glomeruli of the GH animals by competitive PCR of isolated microdissected glomeruli. This upregulation persisted late in the course of the disease. We have started exploring therapeutic manipulations using angiotensin converting enzyme inhibitors (ACE I) and non-anticoagulant heparin. The effects of treatment were followed by morphology, and competitive PCR for type IV collagen, type I collagen, smooth muscle actin and laminin B1 mRNAs. The course of the glomerular lesions was unaltered following the former therapy and there appeared to be a worsening of vascular lesions presumably due to a local increase of renin. In addition there was no decrease in mRNAs coding for any of the genes listed above. In contrast, the lesions were reduced by light microscopy and the mRNAs coding for several extracellular matrix components decreased substantially following two weeks of heparin treatment. The nature of the heparin effect is currently being investigated in further detail since this molecule or other related species could be proposed in the treatment of intractable progressive human glomerulosclerosis for which there is presently no effective therapy.



PERIOD COVERED

10/1/92 thru 9/30/93

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of IGF-I in the Biology of Mesangial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: LJ Striker, Chief RCBS

Others: G Striker, Senior Investigator

S Elliot GS12

Ci-Jiang He, Visiting Associate

EP Peten, Visiting Associate

COOPERATING UNITS (if any)

M Hattori, Joslin Institute, Harvard Medical School, Boston.

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology

INSTITUTE AND LOCATION

NIDDK, Bldg 10, Room 3N-110 Bethesda, MD 20892

TOTAL STAFF YEARS:

0.30

PROFESSIONAL:

0.30

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The IGF-I axis has been implicated in the development of renal complications of diabetes. Glomerulosclerosis of diabetes is characterized by mesangial cell proliferation and accumulation of extracellular matrix in the mesangium. This suggests that there may be an intrinsic defect of mesangial cell behavior. Non-obese diabetic mice (NOD) develop glomerular lesions early after the onset of IDDM. We developed new lines of mesangial cells derived from these animals to study the role of the IGF-I axis in a model of spontaneous diabetes. We examined the IGF-I receptor, IGF-I production, and IGF-I binding proteins of the mesangial cells obtained from NOD mice and compared before and after the appearance of diabetes. The cells derived from the diabetic mice exhibited an accelerated turnover. We previously demonstrated the presence of IGF-I receptors and the synthesis of IGF-I in normal glomerular mesangial cells. [<sup>125</sup>I]IGF-I specifically bound to the cell surface of all cell lines. Cross-linking studies showed a single band of radioactivity with an estimated mol.wt. of 125kD, consistent with the  $\alpha$ -subunit of the IGF-I receptor. Radiolabelled IGF-I was not degraded by either cell types. The NOD cells produced more IGF-I after the appearance of diabetes but exhibited less surface receptors. These findings suggested that diabetes induced phenotypic changes in the autocrine loop of IGF-I in the glomerulus.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43228-06

PERIOD COVERED

10/1/92 thru 9/30/93

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology of Human Mesangial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: GE Striker, Senior Investigator

Others: LJ Striker, Chief RCBS

S Elliot, GS 12

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology

INSTITUTE AND LOCATION

NIDDK NIH Bethesda, Md 20892, Bldg 10 Room 3N-110

TOTAL STAFF YEARS:

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human X (b) Human (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project is currently inactive.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43231-05 MD

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Idiopathic Membranous Nephropathy

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	H. A. Austin	Medical Officer	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	K. MacKay	Expert	MDB, NIDDK

COOPERATING UNITS (if any)

Stanford University, Stanford, CA (Dr. B. Myers); CC (E. Vaughan, Nursing); Armed Forces Institute of Pathology; Washington, DC (Drs. T. Antonovych and S. Sabnis).

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.4

PROFESSIONAL:

0.4

OTHER:

CHECK APPROPRIATE BOXES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☒ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Patients with idiopathic membranous nephropathy are being studied to evaluate the efficacy and toxicities of the addition of intermittent cyclophosphamide to alternate day oral corticosteroid therapy. Efficacy will be judged by determinations of effective renal plasma flow, glomerular filtration rate and glomerular capillary wall permselectivity performed with dextran and urine protein (albumin and immunoglobulin) clearance techniques. Kidney biopsy morphology (including morphometric analysis) will be examined at the beginning and at the end of treatment as part of detailed studies of structure-function relationships and the efficacy of various therapeutic modalities.

Patients with membranous nephropathy and 2 or more grams per day of proteinuria will be treated with alternate day prednisone and will be randomized to receive: a) no additional therapy (control group), or b) intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses. Analysis will include comparison of the number of favorable outcomes of glomerular function and pathology, as well as drug-related toxicities observed in each treatment group at the end of the 12 months of study.







100

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43232-04

PERIOD COVERED

10/1/92 thru 9/30/93

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Renal Lesions in the Ablation Model

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: LJ Striker, Chief RCBS

Others: GE Striker, Senior Investigator

COOPERATING UNITS (if any)

Saulo Klahr, Washington University, St Louis, MO  
Carlo Pesce, Dept of Pathology, Genova, Italy

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology

INSTITUTE AND LOCATION

NIDDK Bethesda MD 20892, Bldg 10, Room 3N-110

TOTAL STAFF YEARS:

0.10

PROFESSIONAL:

0.10

OTHER:

0

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither  
(a1) Minors  
(a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Subtotal nephrectomy in rats, the 5/6ths ablation model, leads to progressive loss of kidney function and chronic renal failure. We postulated that the progressive glomerular lesions were due to an early increase in the turnover of glomerular resident cells. This leads to abnormal glomerular growth, with an increase in the glomerular volume which was detectable using morphometric measurements. We have performed autoradiographic studies, using <sup>3</sup>H-thymidine, and found that within two days following subtotal nephrectomy there was an increase in the glomerular cell mitotic index, as well as an increase in the turnover of the cells in the arterial wall. These findings suggest that dysregulation of cell growth is an early event in the development of glomerulosclerosis following reduction in renal mass.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43234-04 MD

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interactions between TGF- $\beta$  and Glomeruli

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: K. MacKay Expert MDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.80

PROFESSIONAL:

0.80

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Increases in glomerular cellularity and accumulation of extracellular matrix material are prominent histologic findings in a number of clinical and experimental glomerular diseases. Transforming growth factor- $\beta$  (TGF- $\beta$ ) has been identified as a potentially important modulator of glomerular pathology based on its demonstrated ability to regulate proliferation and extracellular matrix synthesis by cultured glomerular cells. In addition, we have previously demonstrated that normal rat glomeruli contain high concentrations of TGF- $\beta$ 1 and TGF- $\beta$ 2 and that glomeruli possess unique TGF- $\beta$  binding proteins or receptors. The goal of these studies is to better understand the actions and mechanisms of action of TGF- $\beta$  in the glomerulus. The current focus of these studies is on TGF- $\beta$  binding proteins and receptors.

We have identified subpopulations of type I and II receptors which have different affinities for TGF- $\beta$ 1 and TGF- $\beta$ 2. Evaluation of TGF- $\beta$  receptors in CHO cells demonstrated that: (1) all of the type I receptors and the majority of type II receptors have a density (determined with sucrose density gradients) which much greater than that predicted by their molecular weight, and (2) the subpopulations of type I and II TGF- $\beta$  receptors with differing affinities for TGF- $\beta$ 1 and TGF- $\beta$ 2 can be separated by sucrose gradient centrifugation. These findings are consistent with the hypothesis that differences in affinities of type I and II TGF- $\beta$  receptors for TGF- $\beta$ 1 and TGF- $\beta$ 2 are due to their participation in different types of receptor complexes.





## PERIOD COVERED

10/1/92 thru 9/30/93

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glomerular Lesions in Non-Obese Diabetic Mice

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: LJ Striker, Chief RCBS

Others: GE Striker, Senior Investigator

C W Yang, Visiting Associate

Ci Jiang He, Visiting Associate

## COOPERATING UNITS (if any)

M Hattori, Joslin Institute, Harvard Medical School, Boston, MA

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, Bldg 10, Room 3N-110, Bethesda, MD 20892

## TOTAL STAFF YEARS:

0.60

## PROFESSIONAL:

0.60

## OTHER:

## CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither

(a1) Minors

(a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

NOD mice spontaneously develop insulin-dependent diabetes mellitus (IDDM) secondary to immunologically-mediated beta cell destruction in pancreatic islets. These mice exhibit an increase in the amount of mesangial matrix before diabetes mellitus occurs and are therefore "prone to glomerulosclerosis". Shortly after the appearance of diabetes, there is an accentuation of the renal lesions consisting of mesangial sclerosis, thickening of glomerular basement membranes, and albuminuria. Morphometric analysis showed that the kidney weight and glomerular size were increased in diabetic mice, compared to non-diabetic mice, and that the ratio glomerular volume/kidney weight was elevated in diabetic mice. We explored by competitive PCR of isolated glomeruli the glomerular turnover of the extracellular matrix components. Prior to the appearance of diabetes the levels of type IV collagen mRNA were 3-4 times higher than in SJL mice who have normal glomeruli. In addition the NOD mice had fewer glomeruli than the SLJ and those were larger. Therefore the NOD strain has a propensity to develop sclerosis irrespective of diabetes which is underlied by an abnormal level of expression of basement membrane gene expression and by a reduced number of glomeruli. We are now investigating whether the occurrence of diabetes mellitus leads to an upregulation of the collagens gene expression. This study is the first to establish that there is a different baseline expression of type IV collagen in different strains of mice and may be directly applicable to man since it is well known that the susceptibility to progressive glomerulosclerosis varies according to the ethnical background.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43236-03

PERIOD COVERED

10/1/92 thru 9/30/93

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glomerular Effects of Advanced Glycosylation End Products (AGEs)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: LJ Striker, Chief RCBS

Others: CW Yang, Visiting associate

GE Striker, Senior investigator

COOPERATING UNITS (if any)

Helen Vlassara, Picower Institute for Medical Research, Manhasset NY

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology

INSTITUTE AND LOCATION

NIDDK, Bldg 10, Room 3N-110, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.60

PROFESSIONAL:

0.60

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

End-stage glomerulosclerosis constitutes a major complication of diabetes mellitus. The fact that the glomerular lesions in both IDDM and NIDDM are similar suggests that abnormalities in glucose metabolism may participate in their development. Hyperglycemia leads to the accumulation of advanced glycosylation end-products. These products participate in abnormal, non-metabolizable cross-linking of extra-cellular matrix components. Their accumulation may contribute to the sclerosis observed in diabetics. AGEs trigger a large number of biological reactions which are mediated by surface receptors that have been characterized on macrophages, endothelial cells, and human and rat mesangial cells. Using normal mouse mesangial cells, we investigated the effect of AGE on the synthesis of the basement membrane components. Cells plated on AGE showed increased levels of the following mRNAs using the RNase protection assay: collagen type IV, proteoglycan heparan sulfate, and laminin A and B chains. We also found an increased release of collagen type IV into the medium. The rate of transcription, measured by nuclear run-off assays, was also stimulated in cells plated on glycosylated bovine serum albumin. AGE receptor antibodies inhibited the observed increase in mRNAs. Antibodies to PDGF abrogated the AGE response. Since these observations were made in vitro we have examined whether the administration of AGEs to the intact animal would have similar effects. The glomeruli of normal mice receiving repeated injections of AGEs exhibited an increase in mRNAs coding for  $\alpha 1$  type IV collagen and for the B1 chain of laminin establishing that there is a glomerular response to AGEs in vivo.

1. The first part of the paper is devoted to a discussion of the general principles of the theory of the structure of the atom. It is shown that the structure of the atom is determined by the laws of quantum mechanics, and that the laws of quantum mechanics are derived from the principles of relativity and the theory of the structure of the atom.

2. The second part of the paper is devoted to a discussion of the general principles of the theory of the structure of the atom. It is shown that the structure of the atom is determined by the laws of quantum mechanics, and that the laws of quantum mechanics are derived from the principles of relativity and the theory of the structure of the atom.

3. The third part of the paper is devoted to a discussion of the general principles of the theory of the structure of the atom. It is shown that the structure of the atom is determined by the laws of quantum mechanics, and that the laws of quantum mechanics are derived from the principles of relativity and the theory of the structure of the atom.

4. The fourth part of the paper is devoted to a discussion of the general principles of the theory of the structure of the atom. It is shown that the structure of the atom is determined by the laws of quantum mechanics, and that the laws of quantum mechanics are derived from the principles of relativity and the theory of the structure of the atom.

5. The fifth part of the paper is devoted to a discussion of the general principles of the theory of the structure of the atom. It is shown that the structure of the atom is determined by the laws of quantum mechanics, and that the laws of quantum mechanics are derived from the principles of relativity and the theory of the structure of the atom.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43237-03

PERIOD COVERED

10/01/92 thru 9/30/93

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Production of Metalloproteinases and TIMPs by Mouse Glomerular Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: LJ Striker, Chief RCBS

Others: G Striker, Senior Investigator

S. Elliot

COOPERATING UNITS (if any)

W Stetler-Stevenson, Laboratory of Pathology, NCI, NIH  
M Carome, Walter Reed Army Medical Center

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Renal Cell Biology

INSTITUTE AND LOCATION

NIDDK, Bldg. 10, Rm. 3N-110, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.07

PROFESSIONAL:

0.07

OTHER:

0.01

CHECK APPROPRIATE BOX(ES)

- (a) Human (b) Human X (c) Neither  
(a1) Minors  
(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Glomerulosclerosis occurs in a large number of human kidney diseases, including diabetic nephropathy. It consists of the accumulation of extracellular matrix (ECM) within the glomerulus. Sclerosis may be mediated by dysregulation of both synthesis and degradation of ECM. A large family of matrix metalloproteinases as well as tissue inhibitors of metalloproteinase (TIMPs) play a role in the degradative process. Our preliminary work reveals that: 1) normal mouse mesangial cells in culture secrete a 72 kD and a 92 kD gelatinase (type IV collagenase) as well as TIMP1; 2) mesangial cells derived from NOD mice and mice transgenic for bovine growth hormone (bGH) secrete mostly the 72 kD gelatinase; Normal mouse glomeruli contain mRNA coding the 72KD species. This mRNA is increased in mice transgenic for bGH who have sclerotic lesions. TIMPs mRNAs are undetectable in mouse glomeruli which emphasizes the major phenotypic changes that occur in vivo. In the GH mice the upregulation of the 72KD gelatinase is less prominent than that of type IV collagen. This finding may suggest that there is an imbalance between synthesis and degradation of extracellular matrix in certain forms of glomerulosclerosis.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43238-03

PERIOD COVERED

10/01/92 thru 9/30/93

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gene Expression in Microdissected Mouse Glomeruli

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: L Striker, Chief RCBS

Others: G Striker, Senior Investigator

E Peten, Visiting Associate

CJ He, Visiting Associate

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology

INSTITUTE AND LOCATION

NIDDK, Bldg 10, Room 3N-110, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.47

PROFESSIONAL:

0.47

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human (b) Human X (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this study is to determine the phenotype of multiple extracellular matrix molecules in normal adult mouse glomeruli. We used the increased sensitivity afforded by the polymerase-chain reaction to assess  $\alpha 1$  type I and several  $\alpha$  chains of type IV collagen mRNA in freshly microdissected normal adult mouse glomeruli. RT-PCR reactions for mRNA encoding these components were also performed using mesangial cell lines previously isolated from the same strain of mice. Type IV collagen mRNA was easily detectable in normal adult mouse glomeruli as well as in the cell lines. On the other hand, type I collagen mRNA was not detected in normal glomeruli, despite increasing the number of PCR cycles from 25-45 (roughly a 1000 fold increase in sensitivity). Assays using competitive PCR were developed. Utilizing the same primers, type I collagen mRNA was easily demonstrable in two lines of mouse mesangial cells. These experiments support data in both humans and experimental models which failed to demonstrate type I collagen by immuno-fluorescence microscopy in normal glomeruli, whereas type IV collagen was present in large amounts. The current study provides evidence that the expression of types I and IV collagen in normal glomeruli is regulated at the pretranslational level in vivo. They also provide evidence for a continuous turnover of basement membrane in the adult glomeruli.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43239-02 MD

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Mechanisms and Modulation of T cell Activation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.:	D. T. Boupas	Visiting Scientist	MDB, NIDDK
Others:	J. E. Balow	Senior Investigator	MDB, NIDDK
	S. S. Ahuja	Visiting Associate	MDB, NIDDK
	F. Paliogianni	Visiting Fellow	MDB, NIDDK
	J. P. Balow	Summer Student	MDB, NIDDK

COOPERATING UNITS (if any)

University of Washington, Seattle (M.A. Valentine)  
Section on Immunology, NIAAA, NIH (R.L. Kincaid)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

1.8

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Activation of T cells is a complex process involving cell membrane, cytoplasmic and nuclear events. These events enable T cells to proliferate and exert their immunoregulatory function. The purposes of these studies are: (a) to better understand the role of tyrosine phosphorylation and the calcium-dependent pathways in T cell activation, and (b) to define the effects of biologic agents used for the therapy of immune mediated renal diseases (glucocorticoids, cyclosporine A), as well as those produced at the site of inflammation (prostaglandin E<sub>2</sub>, transforming growth factor- $\beta$ ), on T cell activation.

Glucocorticoids specifically inhibit the tyrosine phosphorylation of protein(s) of 100 kDa in response to stimulation via TCR. Glucocorticoids inhibit the activity of IL-2 promoter by interfering with the activity of nuclear factors NF-AT and AP-1 which are essential for IL-2 promoter, while cyclosporine A (which also inhibits IL-2 promoter activity) interferes with NF-AT activity. Both glucocorticoids and cyclosporine A inhibit Ca<sup>2+</sup>-- but not PKC-dependent pathways of T-cell activation. Both agents inhibit calcineurin activity, whereas glucocorticoids also inhibit CaM kinase activity. Finally, glucocorticoids inhibit both the initiation and the progression components of T cell cycle whereas TGF- $\beta$  predominantly inhibits the late progression phase.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43240-02 MD

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transcriptional Regulation of Immunoglobulin Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P. I.:	D.T. Boumpas	Visiting Scientist	MDB, NIDDK
Others:	F. Paliogianni	Visiting Fellow	MDB, NIDDK
	J. P. Balow	Summer Student	MDB, NIDDK

COOPERATING UNITS (if any)

None

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.5

PROFESSIONAL:

0.4

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Reduction of pathogenic autoantibodies is the main objective in the therapy of immune mediated renal diseases. Although rates of immunoglobulin synthesis can be regulated at multiple levels, most attention has been focused on transcription, as this seems to be the limiting step in most situations which have been examined.

The goal of these studies is: (a) to examine the role of calcium-calmodulin dependent protein kinases and phosphatases that are induced upon activation of B cells in regulating the transcription of immunoglobulin genes, and (b) to examine the ability of a variety of agents (known to regulate the activation, proliferation and differentiation of B cells) to modulate the function and the nuclear transcription of immunoglobulin heavy and light chain genes. These agents include antibodies to surface immunoglobulin, cytokines (transforming growth factor), bacterial mitogens, pharmacologic agents and immunosuppressive drugs.

The mechanism of action of these agents will be explored by examining their effects on the rate of transcription of immunoglobulin genes. Particular emphasis will be placed in detecting similarities, differences, synergism or antagonism in their effects.

1900

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43241-02

PERIOD COVERED

October 1, 1993 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Pathogenesis of Glomerulosclerosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: LJ Striker, Chief RCBS

Others: GE Striker, Senior Investigator

EP Peten, Visiting Associate

CJ He, Visiting Associate

A Patel, GS-11

COOPERATING UNITS (if any)

M Carome, Walter Reed Army Medical Center

International Collaborative Group for Molecular Studies of Kidney

Biopsies (see attached list)

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology, Bldg. 10, Rm. 3N-110

INSTITUTE AND LOCATION

NIDDK, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

0.60

OTHER:

0.40

CHECK APPROPRIATE BOX(ES)

- (a) Human ☒ (b) Human ☐ (c) Neither ☐  
(a1) Minors ☐  
(a2) Interviews ☐

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Most progressive glomerular diseases leading to ESRD have increased extracellular matrix within mesangial areas. Elucidation of the nature and the rate of ECM accumulation in normal and diseased states are prerequisites for the understanding and possibly interfering with progressive glomerulosclerosis. This was the rationale to undertake detailed studies addressing these issues in normal subjects and patients. Since glomeruli represent a small fraction of the renal cortex and since changes in their size and matrix synthesis are regulated independently of other cortical elements, changes in whole kidney preparations do not accurately reflect those in glomeruli. We developed a technique consisting of microdissection of glomeruli, in situ RT of mRNA into cDNA, and PCR. To quantitate small changes in collagen type IV mRNA expression, we developed a competitive PCR assay, which has a level of sensitivity (0.01 to 0.1 attomole) allowing quantitation of the  $\alpha 2IV$  collagen cDNA expressed in a fraction of a glomerulus. To see if this method applied to human diseases, we examined nephrectomies performed for renal carcinoma since a large proportion of these patients have glomerulosclerosis. We found that  $\alpha 2IV$  collagen cDNA was significantly elevated (3.8x) in 5 patients with glomerulosclerosis compared to 5 patients with normal glomeruli. The cDNA increase was not paralleled by cell number in the sclerotic glomeruli. We have started a collaboration with a multi-center group in order to examine either cDNA or isolated microdissected glomeruli obtained from biopsies done for diagnostic purposes in order to develop markers of progression in human diseases with a focus on diabetes.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43242-02

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Degradation of Extracellular Matrix in Human Glomeruli

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title,

PI: LJ Striker, Chief RCBS

Others: GE Striker, Senior Investigator

EP Peten, Visiting Associate

COOPERATING UNITS (if any)

MA Carome, Walter Reed Army Medical Center

WG Stetler-Stevenson, NCI, Laboratory of Pathology

LAB/BRANCH

Metabolic Diseases

SECTION

Renal Cell Biology

INSTITUTE AND LOCATION

NIDDK, NIH Bldg 10 Room 3 N110 Bethesda MD 20892

TOTAL STAFF YEARS:

0.15

PROFESSIONAL:

0.15

OTHER:

CHECK APPROPRIATE BOX(ES)

(a) Human X (b) Human (c) Neither

(a1) Minors

(a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Glomerulosclerosis is characterized by a net accumulation of extracellular matrix components in the glomerulus and results in its progressive obliteration. We started to investigate whether this condition could result from an imbalance between synthesis and degradation of the extracellular matrix. The metalloproteinases which degrade basement membrane collagens are inactivated by a series of inhibitors: TIMPs (tissue inhibitors of metalloproteinases). We examined whether TIMPs mRNAs and the corresponding peptide were expressed in normal and sclerotic glomeruli. We utilized the non tumoral part of nephrectomies performed for cancer in a series of 10 adult patients. The glomeruli were microdissected and reverse-transcribed in situ. Competitive polymerase chain reaction was performed for TIMP I and II mRNAs. Both were detected in the glomeruli and were increased in the 4 specimens in which glomerulosclerosis was present. The increase was not due to an increase in the number of cells in the glomeruli with sclerosis. These data suggest that TIMPs are increased on a per cell basis in the glomerulosclerosis associated with carcinoma. Further studies are in progress to determine whether this increase is also observed in other forms of human glomerulosclerosis. We have also obtained preliminary data indicating that mRNA coding for the 72kd metalloproteinase is expressed in human glomeruli.





DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43243-01

PERIOD COVERED

October 1, 1992 through September 30, 1993

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Co-stimulatory Signals for T-cell Activation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	D.T. Boupas	Visiting Scientist	MDB, NIDDK
Others:	J.E. Balow	Senior Investigator	MDB, NIDDK
	S.S. Ahuja	Visiting Associate	MDB, NIDDK
	F. Paliogianni	Visiting Fellow	MDB, NIDDK

COOPERATING UNITS (if any)

Laboratory of Experimental Immunology, FCRP, NCI (H.A. Young)

LAB/BRANCH

Metabolic Diseases Branch

SECTION

Kidney Disease Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

0.8

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

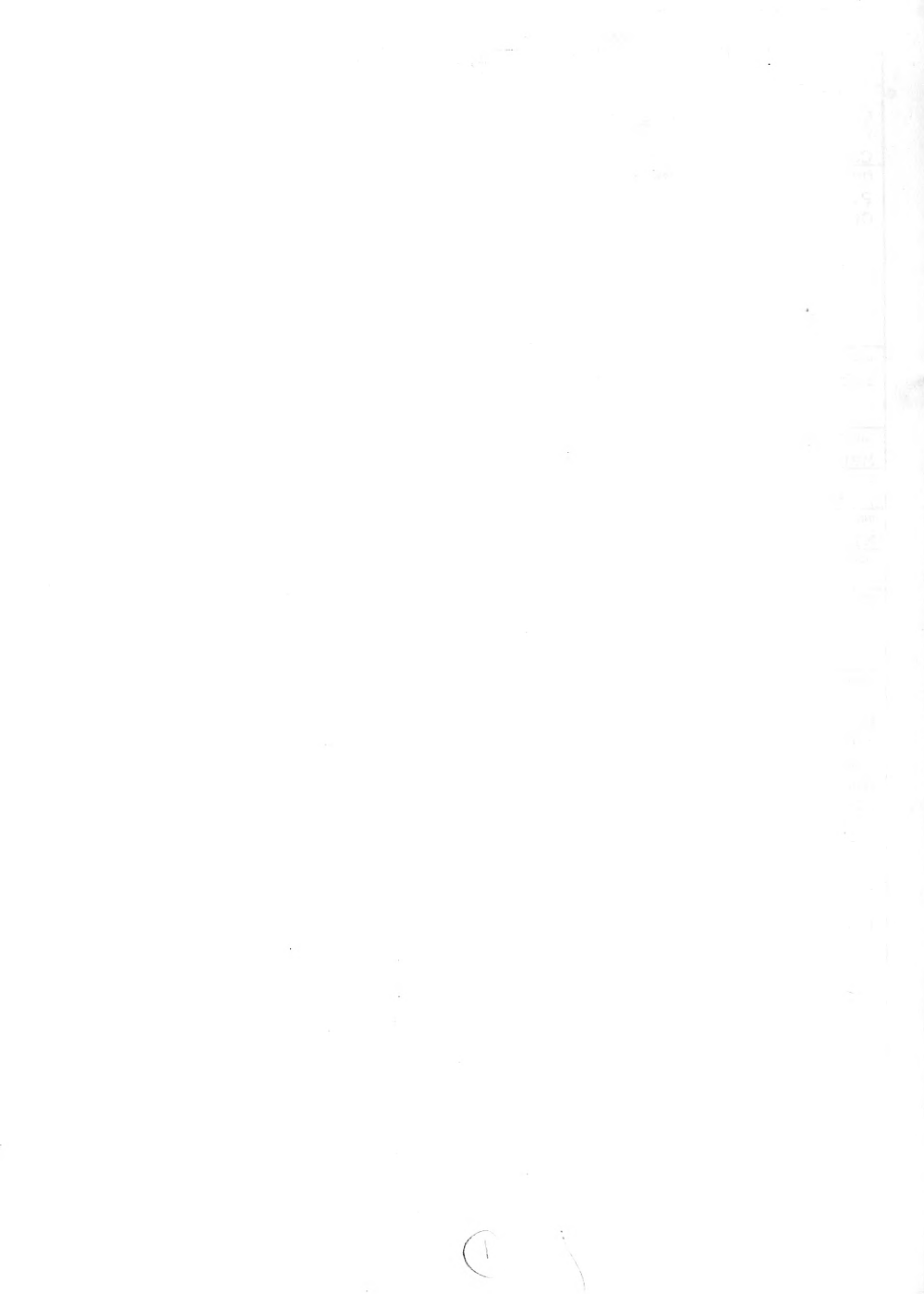
- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

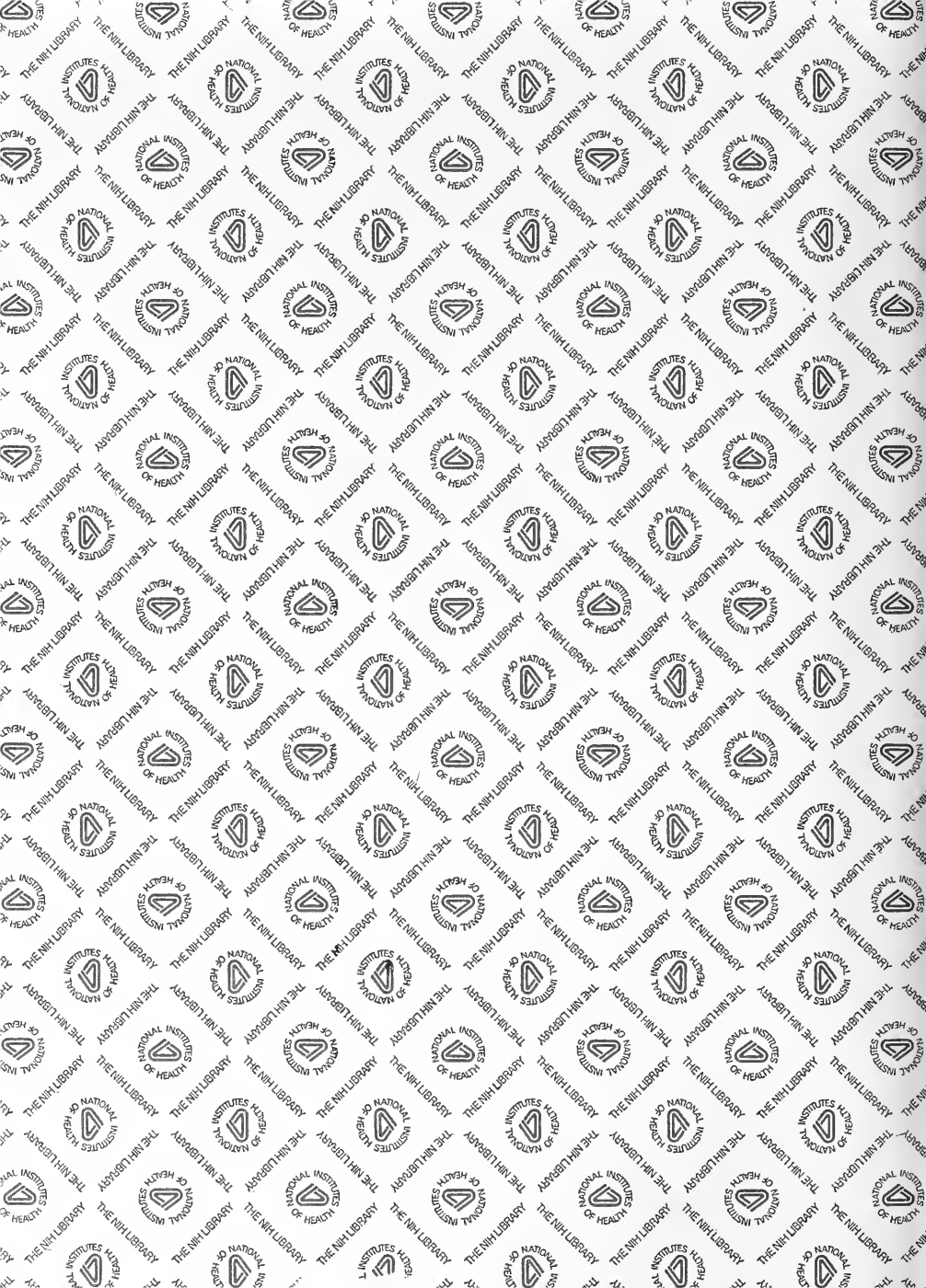
Molecules that interact with the antigen-specific receptor on T-cells, including peptides bound to major histocompatibility complex (MHC) molecules, antibodies against the antigen specific receptor, and mitogenic lectin, fail on their own to stimulate T cells to proliferate. At least one additional signal is required. This second signal is referred to as co-stimulation, and it is delivered by antigen-presenting cells.

The molecular nature of co-stimulation has been sought for many years in order to facilitate therapeutic strategies for suppressing immune responses. The goal of these studies is to further dissect the molecular mechanisms of co-stimulation by the use of naturally occurring immunosuppressive agents.

Findings to date indicate that glucocorticoids inhibit activation of NF-AT and IL-2R $\alpha$  expression but not the tyrosine phosphorylation and calcium influx when cells are stimulated via CD2. IL-2 production is inhibited by glucocorticoids in cells treated with phorbol ester and anti-CD28.









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